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CONTENTS

Calcium and magnesium requirements of Aspergillus niger ..... *Mary Lee Mann* 443

The anatomy of the leaf of Panicum palmifolium ..... *Terzo P. Amidei* 491

Index to American Botanical Literature 500

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Calcium and magnesium requirements of Aspergillus niger

MARY LEE MANN

(WITH THIRTEEN TEXT FIGURES)

INTRODUCTION

Much research has been carried on concerning the relations of calcium and magnesium to the nutrition and various physiological processes of the lower as well as of the higher plants. It is generally agreed that magnesium in small amounts is necessary for the life of all plants, although in only slightly larger amounts it may be toxic to the higher green plants. Calcium has been found to be essential for the life of the higher green plants, but there seems to be some question as to its necessity for the lower forms.

Text books by the following authors serve as excellent sources for the literature on the nutritional requirements of the lower plants: Benecke and Jost (1924), Czapek (1920), Lafar (1911), Lutman (1929), Molisch (1920), Palladin (1926), and Pfeffer (1900). These all concur in the opinion that magnesium is necessary for the nutrition of the lower fungi and that calcium is not essential for these organisms.

A continued article by Tamiya and Morita (1929–1930) appearing in *The Botanical Magazine* of the Tokyo Botanical Society, volumes 43 and 44, gives a bibliography of *Aspergillus* from 1729 through 1928. In all, 2424 papers are listed. This gives an idea of the immense amount of work which has been done by investigators of this organism. Some of the papers which relate to the present undertaking will be cited here.

Nutrient solutions

Raulin (1869) devised a very complicated nutrient solution for fungi. It contained, among many other elements, magnesium, iron, and zinc, but no calcium. A far simpler nutrient solution was that of Pfeffer (1895). This contained, besides a source of carbon, ammonium nitrate, monopotassium phosphate, and magnesium sulphate. These salts were considered sufficient to fulfill all nutritional needs of the fungus. Calcium and iron were not ordinarily included. The same three-salt solution was used by H. Richards (1897), with sucrose as the carbon source and the addition of a trace of iron, for cultivating *Aspergillus niger*.

The question of the necessity of including iron, zinc, and other elements in the nutrient solutions for fungi has been the subject of considerable discussion. Molisch (1892) considered that iron was indispensable for the full growth of *Aspergillus niger* and that spores could not be formed in its absence. Benecke (1895) was not able to prove conclusively the neces-

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sity of iron, while Wehmer (1895) thought that iron was unnecessary for spore formation by this organism. H. Richards (1897) considered that iron was necessary in small amounts for the growth of *Aspergillus niger*. He, moreover, found that iron stimulated the growth of this organism and could be used to replace zinc for this purpose. He, as well as Buromsky (1913), tested the influence of zinc sulphate on this fungus. They both came to the conclusion that zinc does not belong to the indispensable elements, without which the fungus cannot grow, but that it belongs to the substances which stimulate if used in small quantities, but at the same time hinder sporulation. Ono (1900) and H. Richards (1899) found that zinc sulphate stimulated the growth of *Aspergillus niger* and increased the ratio of dry weight to sugar consumed (economic coefficient). Buromsky (1913) also found that zinc sulphate helped the fungus to use sugar economically. The stimulating effect of low concentrations of zinc salts on *Aspergillus niger* was again observed by Richter (1901), who maintained furthermore that copper salts in low and high concentrations always poisoned the fungus.

Coupin (1903a) said that iron, silicon, and zinc have no place in the nutrition of *Sterigmatocystis nigra* (synonymous with *Aspergillus niger*) and that zinc retards development. It was found by Javillier and Sauton (1911) that conidia were formed if iron sulphate was omitted from the nutrient solution and that large amounts of zinc sulphate caused nonsporulation. They concluded that if iron is really indispensable for the formation of spores by *Aspergillus niger*, the amount needed must be extremely small. Waterman (1912) did not consider that a high weight of mycelium is always a favorable indication of the growth of this fungus, but that the power of spore formation must be taken into consideration. He found that certain concentrations of copper sulphate, zinc chloride and sulphate increased the assimilation quotient of carbon, while the observed increase in dry weight was proportional to retarded spore formation. Very dilute zinc solutions had no effect. Copper salts in all tested dilutions counteracted spore formation.

In the work of Currie (1917) every possible precaution appears to have been taken to exclude iron, even in small traces, from the nutrient solution. This author expressed the opinion that iron is not at all necessary for the development of spores of *Aspergillus niger*. Iron added to solutions in which nitrates were used as the source of nitrogen increased the rate of metabolism and weight of mycelium. When ammonium salts were used instead of nitrates, the addition of iron was entirely without effect.

By means of autoclaving with calcium carbonate, Steinberg (1919) considered that he had removed traces of iron and zinc from his nutrient

solution. *Aspergillus niger* made no growth in the solution treated in this manner. Growth was limited when zinc and iron were added separately, but there was a marked increase when both were added together. Therefore he thought that iron and zinc were both needed by this fungus. Steinberg (1920) considered that there may be a partial replacement of iron and zinc by cobalt and uranium, if the former elements are present in not too low amounts.

Bulkewitsch (1922) found zinc sulphate to have no influence on the weight of the felt and the quantity of ammonia formed when *Aspergillus niger* was grown on a peptone medium. He concluded that the well-known growth acceleration due to this salt is specific and only occurs when a few carbon sources are used—for example, carbohydrates and similar compounds. Frouin (1923) found that the suppression of zinc in the nutritive material, if glucose is used instead of sucrose, does not diminish the dry weight of *Aspergillus niger* and that the suppression of iron increases the weight. Glucose, however, is likely to contain impurities (McHargue and Calfee, 1931). That iron seems unfavorable to proteolytic activity of the same fungus, as shown by the power to liquefy gelatine, was observed by Malfitano and Catoire (1924).

Bertrand and Javillier (1911) thought that manganese possesses a favorable influence on the development of *Aspergillus*. Bertrand (1912a) concluded that manganese determines sporulation, but that iron and zinc must also be present, the three being nutritive elements. Bertrand (1912b) thought that the very minute quantities of manganese usually found in commercial salts fulfill the requirements of the fungus for this element.

Several later papers on the heavy metals show that there is some ground for considering four of these as essential. Bortels (1927) removed traces of iron, zinc, and copper from his nutrient solution by adsorption with blood charcoal. His experiments indicated that iron and zinc were necessary for the life of *Aspergillus niger* and that iron and copper were indispensable for the black color of the spores. Metz (1930) found copper necessary for sporulation, and Wolff and Emmerie (1930) considered that this metal was necessary for growth as well as for sporulation. This is in direct contradiction of the earlier work of Richter and Waterman just mentioned, who attributed only a toxic influence to copper.

Roberg (1928) also concluded that iron and zinc were essential and that copper was needed for spore formation of *Aspergillus niger*. His illustrations of *Aspergillus* felts, which have received iron and zinc, are those of typically "stimulated" cultures; these felts appear to be heavy and wrinkled, and they exhibit subnormal spore production. This effect H. Richards (1897) considered as showing stimulation but not nutrition.

Possibly Roberg's cultures were supplied with supra-optimal concentrations of iron and zinc. It would seem that an element to be really beneficial should encourage a healthy development of spores as well as of mycelium. Roberg (1931) found that (1) zinc in small quantities is an absolutely necessary mineral nutrient for *Aspergillus niger* and for several other species of this fungus; (2) in larger quantities zinc acts as a stimulant; (3) still larger quantities act as a poison. If iron is lacking in sufficient amounts, the first unfavorable influence of zinc shows as inhibition of fructification.

Mokragnatz (1931), growing *Aspergillus niger* on Raulin's solution, found that nickel sulphate had a favorable effect on the dry weight of felts when used in low concentrations; cobalt sulphate, however, had no beneficial effect. McHargue and Calfee (1931), growing *Aspergillus flavus* on a more complicated solution than Pfeffer's (including iron and calcium), found that combinations of the optimum concentrations of manganese, copper, and zinc produced greater growth than did any one of the three or than did combinations of any two. These authors consider that in the ordinary synthetic culture solution these elements are usually present as impurities in sufficient quantities for the normal growth of the plant.

Magnesium and calcium for Aspergillus and Penicillium

Benecke and Molisch were the earliest workers to carry on comprehensive experiments with the object of discovering the relations of magnesium and calcium to the growth of *Aspergillus* and *Penicillium* in nutrient solutions. Benecke (1894a) concluded from his experiments that sulphur, phosphorus, potassium, magnesium, and iron are indispensable for the life of all plants, and that calcium is also needed by the green plants, but not by the fungi. He used *Aspergillus niger* and *Penicillium glaucum* in some experiments in which magnesium sulphate was present in the nutrient solution. The addition of 0.05 per cent of calcium sulphate did not improve the weight or conidium formation. Benecke (1895) grew *Aspergillus niger* in a basic solution containing no magnesium. He tried the effects of adding calcium nitrate, barium nitrate, strontium nitrate, magnesium sulphate, and magnesium sulphate and calcium nitrate together. Only under the last two conditions did he obtain growth resulting in spore-bearing felts. He concluded that magnesium is absolutely indispensable and cannot be replaced by calcium (also Benecke, 1894b). The latter element, if present, may be used by the fungus in an indirect manner, as possibly in the thickening of the cell wall; but it is not an integral substance for the life of the protoplasm as is magnesium. Benecke (1896) tested the effect of small amounts of magnesium sulphate on the same fungus by adding this

salt to 25 cc. of his solution (which contained ammonium sulphate as another source of sulphur) in quantities varying from 0.01 gram to 0.0000025 gram and finally none. The dry weight of fungus formed decreased in direct ratio with the decrease in magnesium sulphate. With none of this salt, the weight was very slight.

Molisch (1894a, 1894b) considered magnesium to be an essential element for fungi as well as for the higher green plants. He proved that *Penicillium sp.* and *Aspergillus niger* did not grow if magnesium sulphate was left out of the nutrient solution, even if calcium sulphate were supplied. He also concluded that calcium cannot replace magnesium and that calcium is not necessary for the growth of the lower fungi, which differ in this manner from the higher green plants.

Wehmer (1895) thought that calcium was wholly out of place in culturing fungi, but was undecided as to the necessity of magnesium. Günther (1897) found the same elements necessary for fungi as were included by Pfeffer in his solution, and decided that magnesium salts could not be replaced by the salts of calcium or related elements. Javillier (1913) considered that magnesium is indispensable for *Sterigmatocystis nigra* and that it cannot be replaced by glucinium. Coupin (1903b) found that magnesium may be used by this fungus as phosphate, sulphate, chloride, nitrate, etc.

The ratio between magnesium and phosphorus was regarded by Reed (1907) as important for the proper development of *Aspergillus niger*. Too large a supply of magnesium as compared with the phosphorus increased the weight of mycelium, but hindered spore formation. Waterman (1913), working with the same fungus, found that comparatively large amounts of magnesium checked the formation of mycelium while still larger concentrations allowed growth only after several days. He thought that very small amounts of zinc might activate magnesium. Canals (1929) decided that magnesium plays a rôle in the life of plants as important as that of carbon, hydrogen, and oxygen. He considered that it probably circulates as magnesium phosphate, which is easily dissociated. The magnesium is thus readily obtainable for making complex molecules necessary for the life of plants, such as chlorophyll in the higher plants and sucrase in the fungi. Rippel and Behr (1930) thought that the mycelium of *Aspergillus niger* contains organic magnesium compounds not soluble in acetone, and that in this respect there is a difference between the fungus and etiolated parts of green plants.

A very ingenious theory was evolved by Loew (1892, 1895, 1899, 1903, 1925) to explain the functions of magnesium and calcium in plants. He held that the element magnesium is needed to carry phosphoric acid to

the cell for the formation of nucleo-proteins and lecithin. Magnesium cannot be replaced by calcium, as this latter element cannot act in this manner. He also supposed that calcium-protein compounds enter into the composition of the nucleus and chlorophyll bodies of the higher plants and higher algae, but that the nucleus of the fungi has a different chemical composition. He thought that by placing various plants in solutions of potassium oxalate any calcium present in the cell would be precipitated. As the lower fungi and lower algae are not harmed by this treatment, Loew considered that they do not need calcium, while *Spirogyra* and many other plants are harmed and consequently do need calcium. Hori (1910), using the same method, decided that *Aspergillus niger*, *Aspergillus flavus* and *Penicillium glaucum* do not need calcium.

Robert (1911, 1912) found calcium present as an impurity in the sugar and salts that she obtained from commercial sources. She grew *Aspergillus niger* with small amounts of calcium sulphate added to Raulin's solution, which was made with materials purified until they gave no reaction for calcium with the chemical tests that she employed. *Aspergillus* grew as well with as without the addition of these small amounts, and all of the calcium added was found in the ash of the mycelium. But when very large amounts of calcium were added, the felt weighed slightly more and only eighty per cent of the calcium was found in the ash. She thought that the calcium was fixed by the oxalic acid formed and that this oxalate in the mycelium accounted for the increase in weight. Wehmer (1906) added calcium carbonate to a three-salt solution and collected many oxalate crystals from the under side of the felt of *Aspergillus niger*.

Steinberg (1919, p. 366) concluded from his experiments that calcium has no effect on the growth of *Aspergillus niger*. A series of tests performed by Buromsky (1913) with the same organism, grown in Pfeffer's solution to which calcium sulphate was added and from which magnesium sulphate was omitted, led him to conclude that calcium has no meaning as a nutrient for this fungus, since growth was very poor under these conditions, and equally so whether ammonium sulphate or nitrate was used as a source of nitrogen. He stated that magnesium serves not only as an element necessary for growth but also as a stimulant, since the dry weight and the ability of the fungus to use sugar economically were increased together by the use of this element, as is true when zinc is used. The same worker obtained very favorable results with calcium and magnesium together, but attributed these entirely to the presence of the magnesium. He also thought that ammonium nitrate is a stimulant, as is zinc sulphate.

Haenseler (1921) compared the effects of sodium nitrate and calcium nitrate on the growth of *Aspergillus niger*. The cultures with calcium gave

uniformly higher yields than did those with sodium having equivalent amounts of NO_3. Thus the calcium ion seemed better for the fungus than the sodium ion. Young and Bennett (1922) used nutrient solutions in which potassium nitrate was compared with calcium nitrate as a source of nitrogen for the growth of *Aspergillus niger* and a number of other fungi. Each solution contained another potassium salt, which supplied the essential potassium ion. They thought that calcium exerted a stimulative action on the growth of the fungi, because the calcium nitrate was more favorable than the potassium nitrate. They also considered that calcium was necessary to counteract the acidity of the solution. Contrary to the great mass of evidence cited above, they say that a salt containing calcium should have a place in the best synthetic solutions for fungi.

Coupin (1927), like Waterman (1912), cast doubt on the conclusions drawn simply from a comparison of dry weights of fungus felts. He found that *Penicillium glaucum*, grown on Raulin's solution containing no calcium, did not produce spores unless a salt of this element was added. But *Sterigmatocystis nigra* seemed indifferent to the presence or absence of calcium. Davis, Marloth, and Bishop (1928) reported that calcium increased the yield of *Aspergillus niger* and *Penicillium italicum* and that spore formation depended upon the presence of this ion. Full details of their experiments have not yet been published.

Magnesium and calcium for other plants

Mayer (1869) studied the fermentation power of yeast in nutrient solutions. His salts were fewer in number than those employed by Raulin. The mineral requirements for continued normal appearance of the yeast cells were fulfilled by potassium phosphate, magnesium sulphate, and calcium phosphate, added to sugar and an ammonium salt. He was doubtful as to whether both ions of magnesium sulphate are essential, and considered that there should be further experiments to settle the necessity of calcium.

Winogradsky (1884) decided that magnesium was absolutely necessary for the growth of *Mycoderma vini*, while calcium was regarded as useless. Molisch (1895) found that some algae behave like the lower fungi in that they can dispense with calcium, while *Spirogyra* and *Vaucheria* die if deprived of this element. Benecke (1898) concluded that these two algae must have magnesium in order to live, and that *Chlamydomonas* and *Protococcus* can live and reproduce vegetatively without calcium.

Loew (1898) thought that bacteria and yeast, since they are not harmed by neutral oxalate solutions, do not need calcium. Loew (1901, 1913, 1925) observed that *Spirogyra* was harmed by oxalate solutions and concluded that this alga needs calcium. Hori (1910) found that several of the

higher fungi would not grow in oxalate solutions; he regarded calcium as indispensable for them. Pringsheim (1926) grew various algae and mosses in solutions of sodium oxalate. He stated that it may be taken for granted that traces of calcium can be absorbed in the presence of oxalate. But his experiments led him to conclude that in general the higher algae and mosses need calcium and the lower algae do not.

Gabritschewsky (1902) did not wholly agree with Loew in regard to the relation of bacteria to calcium. He considered that the value of calcium may vary for different bacteria and expressed the opinion that certain kinds do require calcium. O. Richards (1925) found that calcium sulphate in suitable concentration gave optimum growth of yeast. Small amounts were not sufficient for optimum growth, and larger amounts were toxic.

Dunn (1921), in her study of two races of *Rhizopus nigricans* grown on a three-salt solution with dextrose and a trace of iron phosphate, found that the plus and minus strains made excellent growth if given ammonia, either with or without the addition of calcium. Calcium was not injurious, nor was an appreciable amount essential for active growth of these two races. Some calcium may have been present in the solution, but none was purposely added to it.

Lundegardh (1924) reported that calcium chloride increased the respiration of *Gibberella Saubinetii* in an acid medium, but that calcium ions had no noticeable influence in neutral solutions. He considered that calcium was not needed as a mineral nutrient for this organism.

Antagonism

Comparatively little work has been done with the lower plants on the ability of certain substances to counteract the harmful effects of others—*i.e.*, on the phenomenon that is usually termed antagonism.

Loew (1899) thought that magnesium poisons the higher plants by displacing the calcium of their nuclei and interfering with the capacity for imbibition belonging to the calcium-protein compounds. An excess of calcium, according to Loew, allows the calcium to enter again into these compounds, and thus counteracts the toxic effect of magnesium. Loew gave this explanation of the often observed antagonistic, or beneficial effect of calcium on plants that have been poisoned by magnesium. The lower plants in Loew's opinion do not have calcium in their nuclei, and hence magnesium does not poison them. Benecke (1907) reported that the toxicity of anions as well as cations for *Spirogyra* could be lessened by the use of calcium. Loew and Aso (1907) thought that potassium salts can retard but not prevent the toxic effects of magnesium salts. They considered that the cause of this retardation of the toxic action by potas-

sium salts is entirely different from the cause underlying the prevention of the toxic action by calcium salts; that it may be due to the formation of double salts with potassium.

Osterhout (1905, 1906, 1907b, 1909) found that fresh water algae were killed by a simple solution of sodium chloride, but that the toxicity of this solution could be inhibited if calcium chloride were added. Studies with marine algae showed that each of the salts of sea water was poisonous in simple solution, but that a mixture of these salts in the proper proportions formed a solution in which the toxic effects of the individual salts were mutually counteracted. This mixed solution was called by Osterhout a "physiologically balanced solution". Some of the salts added to make a solution balanced need not necessarily be nutritive. Thus Osterhout found that sodium and potassium showed great similarity in their toxic and protective or antagonistic effects, but not in their nutritive power. A "nutrient solution" of course contains the salts that are essential for the growth of the plant. If this solution is concentrated, the plants will not thrive in it unless the salts are properly balanced. But a nutrient solution may be made in which the concentrations of the salts are so low as not to exert toxic effects (Osterhout, 1907a).

Lipman (1909, 1910), in testing ammonification by *Bacillus subtilis*, found that there was antagonism between calcium and potassium, magnesium and sodium, and potassium and sodium, but there was none between magnesium and calcium or between sodium and calcium. On the contrary there was a progressive increase in the toxic properties of magnesium and of sodium when calcium was added in increasing amounts to a solution of either one. Loew (1910) thought that this result with magnesium and calcium was to be expected. He states that bacteria in general do not need calcium, and that magnesium in the usual biological concentrations is not toxic to lower fungi and algae; hence these plants do not need any antagonistic element. However, Lipman and Burgess (1914), testing nitrogen fixation, found antagonism between calcium carbonate and magnesium carbonate for *Azotobacter chroococcum*. Greaves (1920) reported antagonism between calcium and magnesium as measured in terms of ammonification and nitrification power of soil. Brooks (1920b), in a study of the respiration of *Bacillus subtilis*, demonstrated marked antagonism between magnesium and sodium chlorides and very slight antagonism between magnesium and calcium chlorides. Other tests by Brooks (1920a) showed a pronounced antagonism between sodium and calcium chlorides.

Winslow and Falk (1923a, 1923b) observed favorable effects with low concentrations of sodium chloride and of calcium chloride, and harmful

effects with large amounts of these salts on *Bacterium coli*. The toxic effect was greatest with both salts together. Thus their results are in agreement with those of Lipman with *B. subtilis*.

Lundegardh (1924) reported that *Gibberella Saubinetii* behaves like the higher plants in showing the antagonistic action of calcium and magnesium. Hawkins (1913) germinated spores of *Glomerella cingulata* in hanging drops in nutrient solutions. When a salt of zinc, copper, lead, or aluminum was added, no germination occurred. The addition of calcium nitrate, magnesium nitrate, or potassium nitrate reduced the toxicity of all the heavy metals except aluminum.

The effects of antagonistic salts on the respiration of *Aspergillus niger* were studied by Gustafson (1920). His results with sodium chloride were contradictory. He believed that this salt has a specific chemical action which tends to stimulate respiration, while its osmotic pressure tends to decrease respiration. Sometimes one and sometimes the other effect prevailed, due to physiological differences in the fungus. He concluded that low concentrations of sodium chloride and of calcium chloride, up to 0.5 M, increased respiration, while stronger concentrations decreased it, due to the osmotic effect. Antagonism was shown by the fact that respiration was normal in certain mixtures—that is, it was neither increased nor decreased. Spores of the fungus did not germinate in 0.5 M sodium chloride plus dextrose, while they did germinate in the same strength of calcium chloride plus dextrose, and in various mixtures of the two salts. This showed, he thought, that a substance may have different effects on respiration from those which it has on growth.

The present paper deals with some of the problems which have not been conclusively settled by the work of the authors cited above. It is concerned with the following problems: (1) the necessity of magnesium and the toxicity of extremely large amounts of this element for *Aspergillus niger* and *Penicillium sp.*; (2) the minimum amount of calcium which may be required by these fungi; (3) the possible antagonistic effects of calcium toward other metals; and (4) improvements in the proportions of the salts used in the three-salt solution of Pfeffer. After a description has been given of the general methods that were employed, the experiments and results will be discussed under the four topics just indicated.

METHODS

This work was begun in the botanical laboratory of Barnard College under the direction of the late Prof. Herbert M. Richards. It was continued and finished in the laboratory of plant physiology at Columbia University under the guidance of Professor Sam F. Trelease, to whom the

writer wishes to express her gratitude for his interest and many helpful suggestions. The *Aspergillus* used in performing the series of experiments described in the ensuing pages is believed to be the same organism which was brought from Pfeffer's laboratory in Leipzig by Professor Richards about thirty-five years ago. Dr. Charles Thom, of the United States Department of Agriculture, to whom a culture of the organism was recently sent for examination, decided that it should be regarded as a morphologically aberrant strain of *A. niger*, which may best be designated as "Richards' strain." A descriptive note kindly supplied by Doctor Thom follows:

> *Aspergillus niger* (Richards' strain). This strain varies from the usual races or strains of *A. niger* in the thin-walled stalks, more than usually stained with aspergillin, vesicles quite brittle, primary sterigmata shorter during the usual growing period, but becoming longer in individual heads in old culture. The measurements found when young were near those given by Amons (Thom and Church, 1926. p. 174) for his strain labelled *A. ficuum*, but there is no way of proving identity. Further, there does not appear to be ground for believing that the name *A. ficuum* belonged to Amons' strain since the organism distributed from Berlin and by Dr. Westerdijk from the "Centralbureau" at Baarn was certainly not this one. Until more comparative studies are made, the separation of varieties or species among these black forms is scarcely profitable.

A few experiments were also performed with a strain of *Penicillium* isolated from the air, stock cultures of which were maintained on malt-agar slants. The stock cultures of the *Aspergillus* were grown an slices of bread in deep Petri dishes.

The basic nutrient solution used was Pfeffer's three-salt solution with sucrose as the source of carbon. No iron, zinc, copper, or manganese was added. If these elements are needed, they were probably furnished in sufficient amounts by impurities in the salts.

Pfeffer's solution

	Grams per liter	*Gram-molecules per liter*
Sucrose ($C_{12}H_{22}O_{11}$)	50.0	0.14612
Ammonium nitrate (NH_4NO_3)	10.0	0.12492
Monopotassium phosphate (KH_2PO_4)	5.0	0.03673
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	2.5	0.01014
Total salt concentration	17.5	0.17179

The sucrose and salts were obtained from different sources during the six years in which the experiments were carried on. Their purity sometimes varied. This might not have been noticed in dealing with higher plants, but *Aspergillus* has been found to be extraordinarily sensitive to certain minute differences in the medium. The presence of certain kinds of im-

purities could be detected by the fact that the control culture was slightly stimulated—that is, the mycelial felts were wrinkled and abnormally heavy, and spore production was diminished. If this occurred, salts from other sources were tried, until the control culture gave a normal result—that is, a flat, black, evenly fruited felt, having a weight of approximately 0.30 gram.

A determination of the hydrogen-ion concentration of the culture solution proved to be useful, in conjunction with the appearance of the felts, as a guide in deciding whether the control culture was normal. The pH value of the solution, after normal growth of the fungus, was 2.8 with metacresol purple and 3.0 with bromphenol blue, using the La Motte standard indicator solutions. An aberrant pH value was indicative of abnormal growth. Stimulation—and sometimes inhibition—of growth could be detected by a lowering of the pH value to 1.8. Decreased growth sometimes was associated with a pH value of 3.2 or 3.4. The pH value of the basic solution was always determined before growing the fungus. It was invariably 4.4.

The water used for the final rinsing of glassware and for making solutions was obtained from a Barnstead still and redistilled through a quartz condenser. It was stored in Pyrex flasks. All glassware was treated with concentrated sulphuric acid, and then thoroughly rinsed with tapwater, with single-distilled water and finally with double-distilled water.

The stock solutions of the salts were made up in the desired concentrations and stored in Pyrex bottles, while the sugar solution was made fresh each time of using. The culture solutions were prepared before each experiment by mixing and diluting the stock solutions, to give the required molar concentrations in the final solution.

The fungus was grown in 150 cc. Pyrex flasks of Erlenmeyer form, stoppered with cotton plugs, on 50 cc. of culture solution. The flasks and solutions were sterilized once, for 30 minutes, in an Arnold steam-sterilizer. The inoculations of the solutions in the flasks were made from the stock culture on bread by means of a platinum loop. Care was taken to avoid inclusion of mycelium with the spores. It was possible to cover the surface of the liquid in each flask completely and uniformly with spores; but of course the number of spores placed in each flask must have varied somewhat. That irregularities in the sowing make no important difference in the weights of the felts, after several days' growth, was shown by Linossier (1919), working with *Oïdium lactis*, and the general uniformity of the dry weights of duplicated cultures in the present experiments showed that serious errors were not introduced by irregularities in inoculation.

After the *Aspergillus* cultures had grown for five days in an incubator

at 34°C., the mycelial felts were placed on weighed filter papers, washed twice with distilled water, dried for three days at 65°C., put into a desiccator, and then weighed. From the total weight was subtracted the weight of the filter paper previously taken, thus giving the weight of the felt. Before the filter papers were weighed, they were dried at 65°C. and placed in a desiccator, so that they were in the same condition as when they were weighed the second time, with the fungus felts. The *Penicillium* cultures were grown for six days at room temperature (about 22°C.). In all other respects they were treated in the same way as the *Aspergillus* cultures.

RESULTS AND DISCUSSION

Necessity of magnesium

Benecke, Molisch, Günther, Javillier and Canals, as mentioned in the introduction to this paper, have all concluded from their investigations that magnesium is essential for the development of *Aspergillus niger*. Nevertheless, it seemed desirable in the present study to make further tests of the influence of magnesium sulphate and of magnesium upon the growth of this fungus as well as upon the growth of a species of *Penicillium*. Two series of experiments were carried out. In the first series, magnesium sulphate was omitted from one culture solution and was added in increasing concentrations to the others. The results are given in table 1 and are plotted as graphs in figure 1.

The *Aspergillus* and the *Penicillium* made practically no growth when magnesium sulphate was absent from the nutrient solution. Only a few

TABLE 1

Growth of Aspergillus niger and Penicillium sp. as related to the concentration of magnesium sulphate in Pfeffer's solution. No magnesium sulphate in basic solution.

CONCENTRATION OF $MgSO_4$	DRY WEIGHT OF MYCELIUM[a]	
	Aspergillus niger	Penicillium sp.
M	*Grams*	*Grams*
Zero	.024	.008
.0000208	.104	.044
.000125	.251	.132
.00025	.278	.143
.0005	.305	.143
.00125	.303	.150
.0025	.322	.152
.005	.328	.149
.01	.333[b]	.154[b]
.02	.379	.162

[a] Each value represents the mean for 10 cultures.

[b] Control culture, supplied with same concentration as in Pfeffer's solution.

scattered white patches of very thin mycelium developed on the surface of the medium from which magnesium sulphate had been omitted; germination of the spores began, but growth ceased after a short time.

That the increase in weight of the felts of both fungi varies directly as the increase in concentration of magnesium sulphate, up to at least twice the concentration of this salt in Pfeffer's solution, may be seen by referring to the graphs of figure 1. Both of the curves ascend steadily up to the point denoting the highest concentration used in this series. This was 0.02 M magnesium sulphate—double the concentration of this salt in Pfeffer's solution. As would be expected, both curves rise more rapidly in the

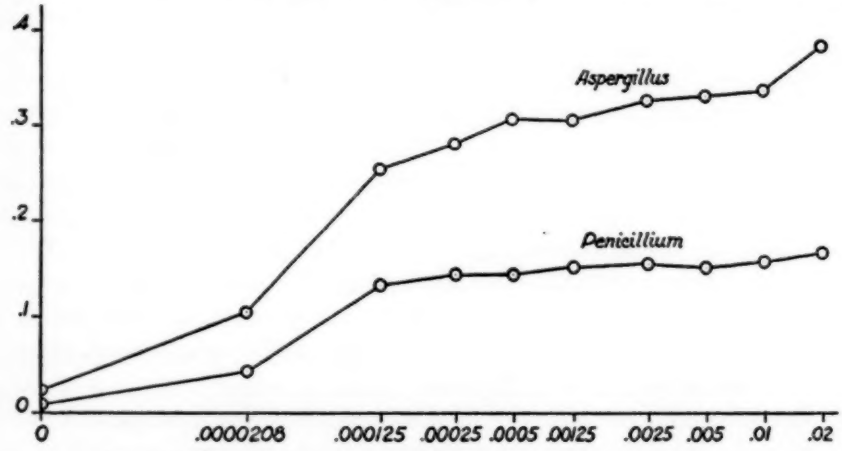

Fig. 1. Dry weights of felts of *Aspergillus niger* and *Penicillium sp.* as related to the concentration of magnesium sulphate in Pfeffer's solution. Magnesium sulphate omitted from basic solution. Abscissas represent molar concentrations of magnesium sulphate (logarithmic scale). Ordinates represent weights of felts in grams. Control culture, supplied with same concentration (0.01 M) magnesium sulphate as in Pfeffer's solution, gave yields of 0.333 gram for *Aspergillus*, and 0.154 gram for *Penicillium*.

lower portion of the concentration range than in the higher. This feature would of course be much more prominent in graphs plotted on an ordinary scale of abscissas than it is in figure 1 in which concentrations are plotted on a logarithmic scale. The results secured in this test are similar to those of Benecke (1896), who obtained higher dry weights of felts of *Aspergillus niger* by increasing the concentration of magnesium sulphate in the nutrient solution.

In the second series of experiments the influence of various concentrations of the magnesium ion was tested by adding magnesium sulphate to a basic solution to which the sulphate ion had been supplied as potassium sulphate. The solution which lacked only the magnesium gave no growth

of *Aspergillus niger*. The results of adding various amounts of magnesium, by means of the same concentrations of magnesium sulphate used in the last experiment, are shown in table 2, and are plotted as a graph in figure 2. It will be noticed that the curves for *Aspergillus* in figures 1 and 2 follow practically the same course. The addition of magnesium sulphate to this solution gave almost the same result as it gave in the first series of ex-

TABLE 2

Growth of Aspergillus niger as related to the concentration of the magnesium ion in Pfeffer's solution. Magnesium sulphate of the basic solution replaced by potassium sulphate.

CONCENTRATION OF $MgSO_4$	DRY WEIGHT OF MYCELIUM[a]
M	*Grams*
Zero	.005
.0000208	.161
.000125	.276
.00025	.274
.0005	.297
.00125	.276
.0025	.288
.005	.312
.01	.336[b]
.02	.362

[a] Each value represents the mean for 5 cultures.

[b] Control culture, supplied with same concentration as in Pfeffer's solution.

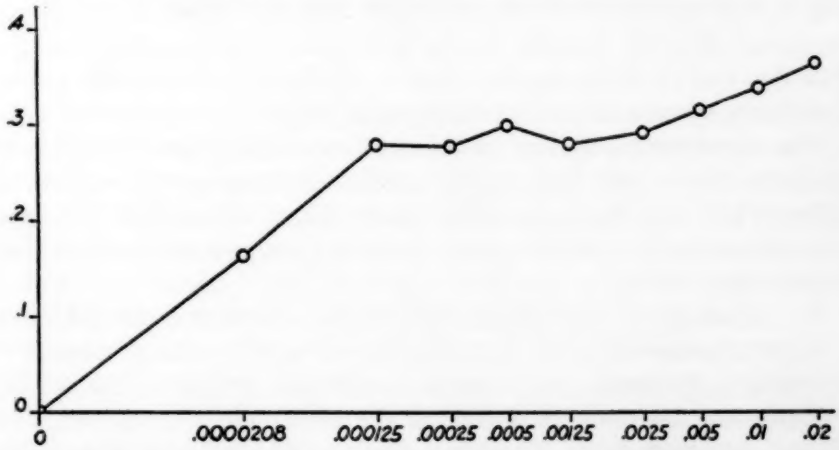

Fig. 2. Dry weights of felts of *Aspergillus niger* as related to the concentration of the magnesium ion in Pfeffer's solution. Magnesium sulphate of basic solution replaced by potassium sulphate. Abscissas represent molar concentrations of magnesium sulphate (logarithmic scale). Ordinates represent weights of felts in grams. Control culture, supplied with same concentration (0.01 M) magnesium sulphate as in Pfeffer's solution (in addition to potassium sulphate) gave yield of 0.336 gram.

periments, in which no potassium sulphate was used in the basic solution. There is a gradually increasing weight of felts as more and more of the magnesium ion is added. It will be observed that in the lower concentrations the curve of figure 1 rises with a slightly steeper slope than that of figure 2. This difference may be attributed to the fact that in the first series both the sulphate ion and the magnesium ion were absent from the basic solution, whereas in the second series only the magnesium ion was omitted from the basic solution.

The close similarity between the parts of the curves corresponding to concentrations of magnesium sulphate greater than 0.00025 M indicates that the higher concentrations of the sulphate ion had little or no influence on the growth of *Aspergillus*.

The second series of experiments was performed only with *Aspergillus niger*, but four cultures of each of the two fungi gave no growth with ammonium sulphate in place of magnesium sulphate. It is therefore clear that the magnesium ion is essential for the growth of both *Aspergillus* and *Penicillium*.

The minimum amount of calcium which may be necessary

Most authors have considered that calcium is unnecessary for the growth of fungi. Benecke, Molisch, Wehmer, Loew, Buromsky, Winogradsky, Steinberg, Dunn, and Lundegardh, as quoted above, have all come to this conclusion. On the other hand, the experiments of Haenseler, Young and Bennett, Coupin, Davis, Marloth and Bishop, Hori, Gabritschewsky, and O. Richards, also cited in the introduction to this paper, have shown some favorable results from its use.

The experiments described in this section of the present paper were planned to throw some light on the question of the minimum amount of calcium which may be necessary for the growth of *Aspergillus*. The tests were of several different types, each of which gave some information concerning this problem.

In one set of tests special precautions were taken to make sure that no calcium was added to the nutrient solution by the water or glassware. Accordingly, the usual procedure was somewhat modified. The double distilled water was redistilled through the same quartz condenser, and collected in quartz flasks. The sugar used was of special purity—Kahlbaum "precip. for calorimetric analysis". The salts and sugar were weighed and dissolved directly in the distilled water contained in a large quartz beaker, and the solution was sterilized by being brought to the boiling point in this beaker. Smaller quartz beakers were used for the culture of the fungi, and quartz pipettes were employed in measuring the water and

solution. Therefore, the only sources of calcium which remained were the salts or the fungus spores.

The weights of the fungus felts of *Aspergillus* and *Penicillium* which were grown in this solution are given in table 3. It will be seen that they are essentially the same as those obtained when these fungi were grown under similar conditions, altered only by the addition of 0.00008 M and 0.001 M calcium nitrate. Moreover, the power of conidium formation was not noticeably modified in either fungus by the addition of these amounts of calcium nitrate.

It is realized of course that this test is not in itself very conclusive, since the salts and the fungus spores probably contained some calcium—more, in fact, than would be derived from the water or glassware. The

TABLE 3

Growth of Aspergillus niger and Penicillium sp. in Pfeffer's solution to which calcium nitrate has been added.

CONCENTRATION OF $Ca(NO_3)_2$	DRY WEIGHT OF MYCELIUM[a]	
	Aspergillus niger	Penicillium sp.
M	*Grams*	*Grams*
Zero	.291[b]	.213[b]
.00008	.287	.194
.001	.302	.185

[a] Each value represents the mean for 20 cultures of *Aspergillus niger* or for 16 cultures of *Penicillium sp.*

[b] Control culture.

salts, however, were of best obtainable reagent or chemically pure grades, and their printed analyses showed only very small quantities of calcium. And the test may be considered to indicate that the amount of calcium required—if any—is exceedingly small in comparison with the quantities of the other elements needed for the growth of these fungi. It will be recalled that Benecke (1894a) obtained no increase of growth by adding calcium sulphate to his solution which already contained magnesium sulphate.

Another test was made in which calcium chloride was added to Pfeffer's solution. The results, shown in figure 6 plotted from the data of table 7, show that the addition of this calcium salt in various concentrations had no appreciable effect on the dry yields of *Aspergillus*. Thus it seems clear that the presence of significantly large amounts of calcium, as the nitrate, sulphate, or chloride, in Pfeffer's solution has no beneficial influence on the growth of *Aspergillus* or *Penicillium*.

In an attempt to obtain further evidence regarding the possibility that calcium might be beneficial to *Aspergillus*, a series of experiments was con-

ducted in which the effects of calcium nitrate were compared with those of the nitrates of sodium, potassium, and ammonium. For this purpose the ammonium nitrate was omitted from the basic solution and replaced by each of these salts in turn, used in varying concentrations. These concentrations were so calculated that the gram-atoms of nitrogen per liter of solution were equivalent. The results are given in table 4 and are shown as graphs in figure 3.

Considering first the graphs for the potassium and sodium series in figure 3, it will be observed that the growth of *Aspergillus niger* with these ions is much less than with equivalent gram-atoms of nitrogen as ammo-

TABLE 4

Comparison of the effects of potassium, sodium, calcium and ammonium nitrates on the growth of Aspergillus niger. Concentration of salts calculated on equivalent amounts of nitrogen. No ammonium nitrate in basic solution.

CONCENTRATION OF KNO_3	DRY[a] WEIGHT OF MYCELIUM	CONCENTRATION OF $NaNO_3$	DRY[a] WEIGHT OF MYCELIUM	CONCENTRATION OF $Ca(NO_3)_2$	DRY[b] WEIGHT OF MYCELIUM	CONCENTRATION OF NH_4NO_3	DRY[b] WEIGHT OF MYCELIUM
M	*Grams*	*M*	*Grams*	*M*	*Grams*	*M*	*Grams*
.01266	.199	.01266	.181	.00633	.183	.00633	.248
.02533	.187	.02533	.187	.01266	.199	.01266	.229
.05066	.205	.05066	.188	.0253	.216	.0253	.247
.1	.212	.1	.196	.05	.260	.05	.253
.248	.211	.248	.190	.124	.269	.124	.284
.4	.215	.4	.200	.2	.237	.2	.310
.8	.224	.8	.188	.4	.232	.4	.371

[a] Each value represents the mean for 15 cultures.

[b] Each value represents the mean for 12 cultures.

Control for KNO_3 series, .370 gram.
Control for $NaNO_3$ series, .362 gram.
Control for $Ca(NO_3)_2$ series, .293 gram.
Control for NH_4NO_3 series, .284 gram.

nium nitrate. There is a slight upward trend in the curve for the potassium nitrate series, but none in that for the sodium nitrate series. The dry weights of the felts at all concentrations of both salts are considerably below those for the control cultures which contained 0.124 M ammonium nitrate; the latter weights were 0.370 gram for the potassium series and 0.362 gram for the sodium series. Also, it was noticed that the conidium formation in the potassium and sodium series was inhibited and that the felts had a brown rather than a black appearance.

The position of the graph for calcium nitrate indicates that this salt is better for the growth of *Aspergillus niger* than potassium nitrate or sodium nitrate. The graph for calcium nitrate starts at about the same

level as the other two and it rises rapidly above them, reaching its highest points at concentrations of 0.05 and 0.124 M. Beyond these it falls again until, at the highest concentration, it lies only slightly above the graph for potassium nitrate. The dry weights of all the calcium nitrate cultures are below that of the control culture (0.124 M NH_4NO_3) for this series—0.293 gram.

Apparently, therefore, only at a range of concentrations from 0.05 M to 0.124 M does calcium nitrate give good growth of *Aspergillus niger* when used under the conditions of the present experiments. Even here it does not give a growth quite equal to that attained by the control culture

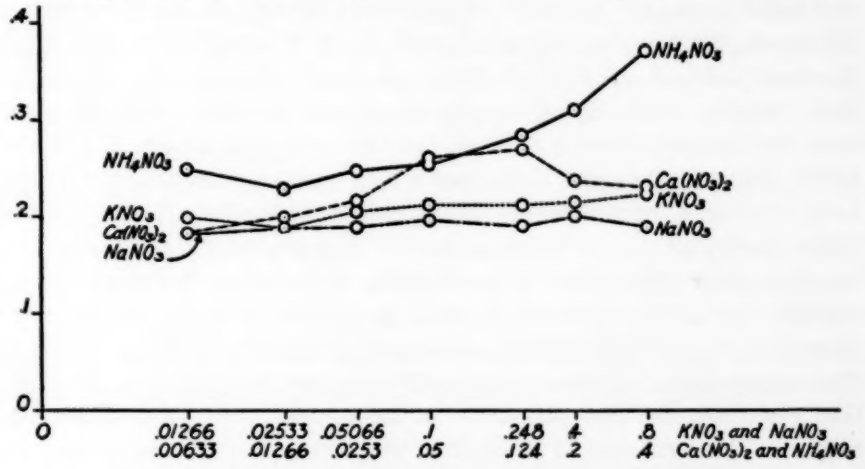

Fig. 3. Dry weights of felts of *Aspergillus niger* grown in Pfeffer's solution to which have been added separately the nitrates of potassium, sodium, calcium, and ammonium. Ammonium nitrate omitted from basic solution. Abscissas represent molar concentrations, plotted as equivalent gram-atoms of nitrogen (logarithmic scale). Ordinates represent weights of felts in grams. Control culture, supplied with same concentration (0.124 M) ammonium nitrate as in Pfeffer's solution gave yields of 0.370 gram for potassium series, 0.362 gram for sodium series, 0.293 gram for calcium series, and 0.284 gram for ammonium series.

grown in Pfeffer's solution. Moreover, conidium formation was inhibited by calcium nitrate, even in this favorable concentration range. The felts were slimy and sank below the surface of the liquid. The culture medium was always observed to be a very deep yellow after the growth of the fungus in the presence of calcium.

It will be seen that the curve for growth with ammonium nitrate, throughout most of its course, lies above the other curves in figure 3. This indicates that ammonium nitrate is much better for the growth of *Aspergillus* than the other three nitrates used in these tests. Only at two points

does the calcium nitrate curve lie near that for ammonium nitrate. At one of these, representing a molar concentration of 0.05, the calcium nitrate curve is slightly above that of ammonium nitrate; and at the next point, 0.124 M, it is nearly as high as the curve for ammonium nitrate. In addition to being heavier, the felts of the ammonium nitrate series had a much healthier appearance, as far as amount and blackness of the conidia were concerned, than had those grown with the other three nitrates. This was true at all tested concentrations.

At the highest concentrations of ammonium nitrate used—*i.e.*, 0.2 and 0.4 M—the fungus made very good growth, giving dry weights of 0.310 and 0.371 gram, respectively. In other experiments during the course of this work, the dry yields obtained with 0.124 M ammonium nitrate (the standard concentration in Pfeffer's solution) often nearly approached these weights. This may be seen by referring to the controls in the potassium and sodium series of table 4, and the controls in tables 1, 2, 6, and 13. It does not seem, therefore, that 0.2 M and 0.4 M ammonium nitrate have acted as a "stimulant" to the growth of *Aspergillus*, if the growth in Pfeffer's solution may be taken as normal. The growth in this solution was found to vary from 0.245 to 0.370 gram dry weight. Buromsky (1913) thought that ammonium nitrate could be compared to zinc sulphate as a stimulant, because ammonium nitrate gave a higher respiration coefficient than ammonium sulphate when used in his nutrient solution. He found that when zinc sulphate was added to the solution containing ammonium sulphate, the coefficient of respiration was raised to almost equal that obtained with ammonium nitrate alone.

On the whole, it may be concluded that calcium nitrate was not very satisfactory as a mineral nutrient for *Aspergillus niger* in this series of experiments, although it gave greater mycelial weights than potassium nitrate, as in the work of Young and Bennett (1922), and greater than sodium nitrate, as in the study by Haenseler (1921). Calcium in no way made up for the absence of ammonium nitrogen, which many workers have reported as the most favorable source of nitrogen for the growth of *Aspergillus*. Boas (1919) and Boas and Leberle (1919) found that an ammonium salt, especially the chloride, was used by *Aspergillus* to a much greater extent than more highly organized nitrogenous compounds offered at the same time. They considered that the ease of dissociation and of entrance into the cell determined the choice of a source of nitrogen by the fungus. Brenner (1914), having tried many sources of nitrogen, concluded that ammonium salts and asparagin gave the best growth of *Aspergillus*.

The experiments described thus far have shown that calcium is not required in any considerable amount by *Aspergillus*, and that relatively high

concentrations of a salt of this element have detrimental, rather than beneficial, effects upon the growth of this organism. There still remains the possibility that very small quantities of calcium are essential for *Aspergillus*. Definite proof that calcium is indispensable could of course be obtained if the calcium content of the culture medium could be sufficiently reduced to prevent the growth of the fungus. In a similar manner, for example, conclusive evidence has been presented during the last decade that small quantities of zinc, manganese, boron, copper, etc., are essential for the growth of many of the higher green plants. It seemed worth while in the present study to reduce the calcium content of the nutrient solution to a very low minimum and to see whether this solution would allow the development of *Aspergillus*. A culture medium of very low calcium content was prepared by adding sodium oxalate to Pfeffer's solution, in concentrations sufficiently high to precipitate practically all of the calcium that might be present as an impurity. The preparation of the culture solution was thus essentially the same as that recommended by Loew (1892, 1898, 1899, 1925), Hori (1910), and Pringsheim (1926).

A test was made of the ability of *Aspergillus* to grow in Pfeffer's solution to which had been added sodium oxalate in the five following concentrations: 0.00625 M, 0.0125 M, 0.025 M, 0.05 M, and 0.1 M. A rather surprising result was obtained, as may be seen from the graph of figure 4, plotted from the data of table 5. The growth of the fungus was greatly

TABLE 5

Growth of Aspergillus niger in Pfeffer's solution containing sodium oxalate.

CONCENTRATION OF $Na_2C_2O_4$	DRY WEIGHT OF MYCELIUM[a]
M	*Grams*
Zero	.284[b]
.00625	.346
.0125	.351
.025	.384
.05	.472
.1	.583

[a] Each value represents the mean for 9 cultures.

[b] Control culture, represents the mean for 18 cultures.

improved by the addition of all tested concentrations of oxalate. The highest concentration used gave very heavy felts. This increased growth may have been due to a stimulating effect of some impurity in the sodium oxalate or to the ability of the fungus to make use of the carbon of sodium oxalate. Since the fruiting was excellent, while fruiting would be inhibited by the typical zinc sulphate stimulation, it seems probable that the greatly

increased weights may have been due to the addition of a second source of carbon for the fungus.

A test was made to find out approximately how much calcium might have been present as an impurity in the nutrient solution employed in the experiment just described. A series of five solutions, having the same composition as those used in the growth test, were freshly prepared. Thus they contained the components of Pfeffer's solution with the addition of

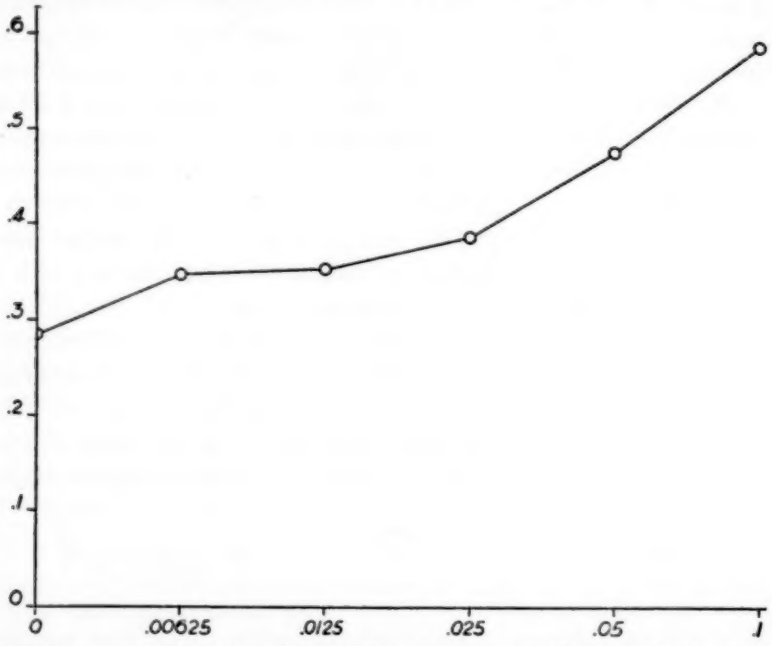

Fig. 4. Dry weights of felts of *Aspergillus niger* grown in Pfeffer's solution to which sodium oxalate was added. Abscissas represent molar concentrations of sodium oxalate (logarithmic scale). Ordinates represent weights of felts in grams. Control culture without sodium oxalate gave yield of 0.284 gram.

0.00625 M, 0.0125 M, 0.025 M, 0.05 M, and 0.1 M sodium oxalate, respectively. Each of these solutions was then tested by adding separately five different concentrations of calcium chloride. The concentrations of this salt employed were 0.0000001 M, 0.000001 M, 0.00001 M, 0.0001 M, and 0.001 M, respectively. This gave a series of twenty-five tests. It was found that the three strongest calcium chloride concentrations (0.00001 M, 0.0001 M, 0.001 M) gave precipitates of calcium oxalate with all of the five oxalate concentrations (0.00625 M, 0.0125 M, 0.025 M, 0.05 M, 0.1 M), and that 0.000001 M calcium chloride gave a visible precipitate with 0.1 M sodium oxalate, but not with the concentrations of oxalate below

this. The lowest concentration of calcium chloride (0.0000001 M) gave no visible precipitate with any of the sodium oxalate concentrations used.

After the fungus had been grown in the same concentrations of sodium oxalate added to Pfeffer's solution, the felts were removed and each of the five culture solutions was tested again, by means of the same five concentrations of calcium chloride. The results were the same as those obtained with the original culture solutions that had been supplied to the fungus. The 0.000001 M calcium chloride solution again gave a precipitate with 0.1 M oxalate.

These tests indicate that, had there been a calcium salt present in a strength of 0.000001 M in the nutrient solution in which *Aspergillus niger* was grown, a precipitation of calcium oxalate would have been observed, since sodium oxalate was present in this nutrient solution in a concentration of 0.1 M. As just pointed out, a precipitate was clearly perceptible when 0.000001 M calcium chloride was added to the solution containing 0.1 M sodium oxalate, both before and after growing the fungus, and no such precipitate was observed in the solution until the addition of calcium chloride. This experiment seems to show definitely that probably less than 0.000001 M of calcium impurities was present in a solution that gave excellent growth of *Aspergillus niger*. This would represent less than 1 part of calcium per 25 million of culture solution. From these tests it may be concluded that, if calcium is indispensable for the growth of *Aspergillus*, the quantity required would be no greater than the amounts of boron, manganese, zinc, and copper that have been found to be essential for many of the higher green plants.

An interesting comparison may be made between the results obtained with *Aspergillus* and those reported by Pringsheim (1926) for various kinds of algae. In one set of experiments Pringsheim first tested numerous algae by the use of solutions as free from calcium as he could make them. He divided these algae into three groups: those which gave no growth without calcium; those for which the deficiency of calcium made little noticeable difference; and those which showed poorer growth but did show some growth without calcium. He then experimented upon the growth of these organisms in a nutrient solution containing sodium oxalate. The culture solution was tested for calcium precipitation, before and after the organisms had grown in it, by the addition of 1 cc. of 0.1 per cent calcium chloride solution. Pringsheim thought, because of the precipitate which he obtained with this concentration of calcium chloride, that a sufficient amount of sodium oxalate was in the nutrient solution to have precipitated calcium from the algal cell had it been present there. The algae which seemed to require calcium in his first experiments did not

grow in the solution containing sodium oxalate. The algae which did not seem to require calcium in his first experiments grew well in the oxalate solutions. There were a few exceptions in which algae which had seemed to need calcium in the first experiments gave favorable results with the oxalate solutions. He accounted for this by saying that their calcium requirements were unusually low, and that enough calcium might have remained in the solution to satisfy these requirements, in spite of the presence of oxalates.

The method of spectrographic analysis furnishes a much more delicate test for calcium than could be obtained by the precipitation method described in the foregoing paragraphs. The chemical precipitation method was only delicate enough to indicate that the culture solution contained less than 0.000001 M calcium. The concentration of calcium in the presence of 0.1 M sodium oxalate may actually have been much smaller than 0.000001 M. It would have been desirable to have had a spectrographic test made upon the mycelium of *Aspergillus* that had been grown in the culture solution containing a high concentration of sodium oxalate, as well as upon a filtered sample of the culture solution itself. But it was not feasible at the time to have this done. One spectrographic analysis, however, was made in the present study, and this was made under control conditions; the fungus was grown in Pfeffer's solution to which no oxalate had been added. This test was made through the courtesy and cooperation of Dr. Andrew Dingwall, of the Department of Chemistry of Columbia University, upon ash obtained from three flat black felts grown in a solution to which no calcium was consciously added in any way. The water was doubly distilled through quartz condensers and the salts were the purest obtainable. The felts, before ashing, were washed many times, using two liters of double distilled water, to remove traces of the culture medium. The ash was found to contain considerable quantities of magnesium and potassium, as might have been expected. In addition there were found small amounts of calcium, iron, manganese, lead, aluminum, and sodium. Other elements may have been present, but a search was not made for them.

The fungus felts may have held these substances in two ways: (1) as materials retained between the hyphal threads by occlusion, (2) as actual components of the fungus cells. Considering the compactness and smoothness of the felts, it seems unlikely that significant quantities of the medium were occluded. The fungus itself probably contained these minute quantities of various elements. They may have been obtained from the bread on which the stock cultures were grown and stored in the spores. Aso (1900) found calcium among the minerals of the ash from the spores of *Aspergillus*

oryzae. Or these elements may have been secured by absorption from impurities in the salts dissolved in the liquid medium. It is of course known that many plants absorb and accumulate substances that are present in extremely low concentrations in the medium in which the plants live. A striking example is furnished by the accumulation of iodine in the giant kelps of the Pacific Ocean.

Effect of calcium chloride in solutions containing certain other salts

That the toxic effect of one cation may be more or less neutralized by the antitoxic effect of another, when they are supplied together in a culture solution for plants, has been observed by many investigators. Some of the pioneer work in this field has been carried on by Loew and Osterhout, as discussed in the introduction to this paper. Mention may be made here of the more recent work of Trelease and Trelease (1926) and Eisenmenger (1928), who conducted experiments which showed that wheat roots are poisoned by relatively high concentrations of a magnesium salt, when grown in a solution of this salt alone. Their results showed that this toxicity may be counteracted by the addition of a calcium salt. A clear demonstration of antagonistic action is obtained most readily with very simple solutions, containing only two salts. In a culture solution containing several salts, the antagonistic relations are very complicated, owing to the chance for the mutual interaction of a large number of ions. But in some cases the antitoxic action of calcium is so pronounced that it can be easily demonstrated with the mixed solutions used for growing the higher plants. Thus, magnesium injury of wheat has been shown to depend upon the ratio of calcium to magnesium in the culture solution (Trelease and Trelease, 1931.)

A series of experiments was carried out with *Aspergillus niger* to see whether calcium, when added to Pfeffer's solution, would have an an-

TABLE 6

Growth of Aspergillus niger in Pfeffer's solution containing magnesium chloride.

CONCENTRATION OF $MgCl_2$	DRY WEIGHT OF MYCELIUM[a]
M	*Grams*
Zero	.339[b]
.2	.331
.4	.334
.6	.236
.8	.223
1.2	.130
1.6	.129

[a] Each value represents the mean for 6 cultures.

[b] Control culture.

tagonistic effect toward other substances present in the culture solution. One set of tests was planned to determine whether magnesium chloride would be toxic for *Aspergillus* when used in high concentrations in the solution; and if so, whether the addition of a calcium salt would counteract the toxicity.

Magnesium chloride was added to Pfeffer's solution in concentrations varying from 0.2 M to 1.6 M. The results of this experiment may be seen by referring to the graph of figure 5, plotted from the data given in table 6. It will be noted that the two lowest concentrations used—namely, 0.2 M and 0.4 M—had very little effect on the growth of the fungus. The weight was normal and there was slight fruiting. At concentrations of 0.6 M and

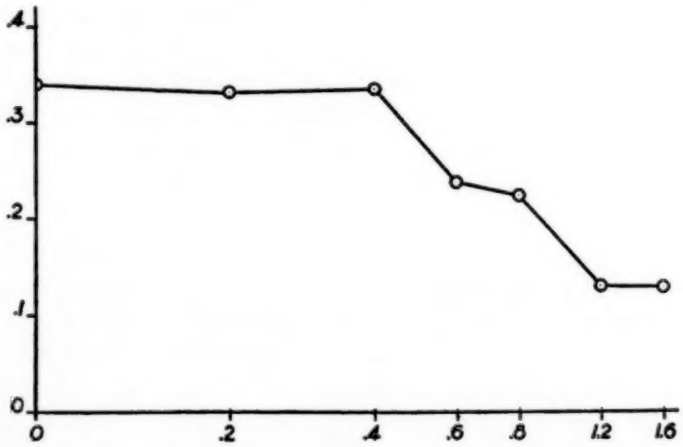

Fig. 5. Dry weights of felts of *Aspergillus niger* grown in Pfeffer's solution to which magnesium chloride was added. Abscissas represent molar concentrations of magnesium chloride (logarithmic scale). Ordinates represent weights of felts in grams. Control culture without magnesium chloride gave yield of 0.339 gram.

upwards, the growth of the fungus was inhibited. The dry weights were consistently reduced, until at 1.2 M and 1.6 M magnesium chloride they were only 38 per cent of the control dry weight of 0.339 gram. The appearance of these last felts was very far from normal. They were white and lacy and completely lacking in conidia.

It is therefore clear that magnesium chloride, if supplied in very high concentrations, inhibits the growth of *Aspergillus niger*. This corresponds to the action of high concentrations of magnesium salts on wheat roots, mentioned above. The concentration of the magnesium ion, however, which is necessary to check the growth of the fungus (0.6 M) is actually considerably higher than necessary to show a similar effect on wheat roots.

Magnesium sulphate is definitely toxic to wheat roots at a concentration of 0.001 M, according to Eisenmenger (1928).

Before testing the effect of the magnesium and calcium chlorides together, the latter was added alone to Pfeffer's solution in concentrations ranging from 0.00625 M to 0.1 M. The graph of figure 6, plotted from the

TABLE 7

Growth of Aspergillus niger in Pfeffer's solution containing calcium chloride.

CONCENTRATION OF $CaCl_2$	DRY WEIGHT OF MYCELIUM[a]
M	*Grams*
Zero	.261[b]
.00625	.266
.0125	.284
.025	.272
.05	.282
.1	.265

[a] Each value represents the mean for 9 cultures.
[b] Control culture.

data given in table 7, shows an almost straight, horizontal line. The sporulation of the felts was good at all concentrations. Hence calcium chloride had no effect on the dry weight or fruiting of *Aspergillus niger* at any concentration used.

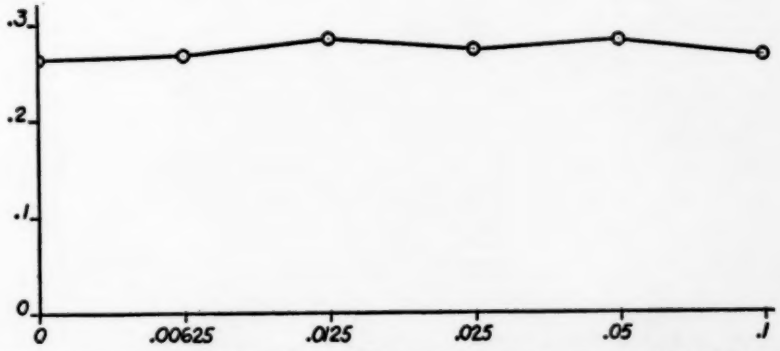

Fig. 6. Dry weights of felts of *Aspergillus niger* grown in Pfeffer's solution to which calcium chloride was added. Abscissas represent molar concentrations of calcium chloride (logarithmic scale). Ordinates represent weights of felts in grams. Control culture without calcium chloride gave yield of 0.261 gram.

Since a concentration of 0.8 M magnesium chloride had been found to inhibit the growth of *Aspergillus* very considerably, this concentration was chosen for use in Pfeffer's solution with various concentrations of calcium chloride. The results of this experiment are shown on the graph

of figure 7, plotted from the data of table 8. It may be seen here that the toxic effect of the magnesium chloride was as great as in the previous test. As the concentration of calcium chloride increases from 0.0001 M to 0.01

TABLE 8

Growth of Aspergillus niger in Pfeffer's solution containing calcium chloride and magnesium chloride.

CONCENTRATIONS		DRY WEIGHT OF MYCELIUM[a]
$MgCl_2$	$CaCl_2$	
M	*M*	*Grams*
Zero	Zero	.297[b]
.8	Zero	.223
.8	.0001	.226
.8	.001	.241
.8	.01	.234
.8	.1	.193

[a] Each value represents the mean for 12 cultures.
[b] Control culture.

M the graph continues practically parallel with the base line. At a concentration of 0.1 M calcium chloride with 0.8 M magnesium chloride, the dry weight was even somewhat lowered. The graph appears to show no significant rise, as it would have shown had there been antagonism. The

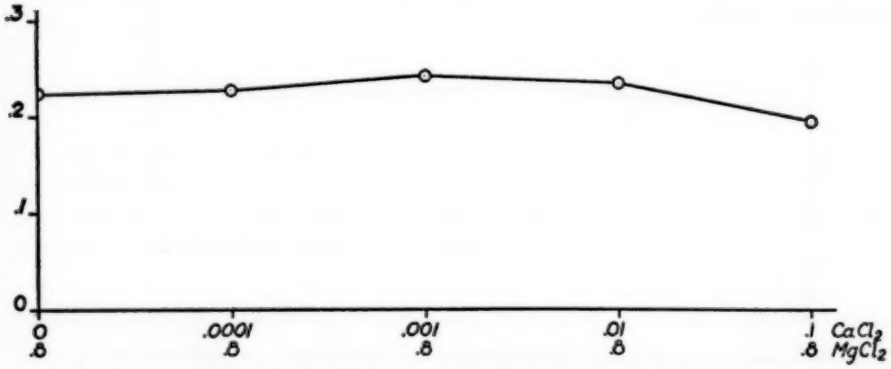

Fig. 7. Dry weights of felts of *Aspergillus niger* grown in Pfeffer's solution containing calcium chloride and magnesium chloride. Each culture contained 0.8 M magnesium chloride. Abscissas represent molar concentrations of calcium chloride (logarithmic scale). Ordinates represent weights of felts in grams. Control culture without magnesium or calcium chlorides gave yield of 0.297 gram.

weights of the felts are all essentially the same as those obtained with 0.8 M magnesium chloride without the addition of any calcium chloride. All of the felts grown in solutions containing both calcium and magnesium chlorides were white, and they were completely lacking in conidia. They

were similar in appearance to those obtained in Pfeffer's solution to which magnesium chloride alone was added. From this experiment it may be seen that the addition of calcium chloride helped neither the growth nor the sporulation of *Aspergillus*, when these were inhibited by a high concentration of magnesium chloride in the culture solution.

Calcium chloride, therefore, seems to have no antitoxic action for magnesium chloride under the conditions of the present tests with *Aspergillus niger*. This lack of antagonism agrees with Lipman's results for *Bacillus subtilis*, previously discussed. But the toxicity of magnesium chloride for *Aspergillus niger* was not increased by calcium chloride (except possibly at a concentration of 0.1 M), as Lipman found to be the case with *Bacillus subtilis*. Loew (1892) stated that magnesium salts in the absence of calcium salts are not harmful to the fungi; he probably referred to low concentrations, as he says later (1910) that they are not harmful in the usual biological concentrations, and that hence there is no need of an antagonistic element. It was only possible to obtain a toxic effect on *Aspergillus* by using concentrations of magnesium chloride which are far higher than those used in most nutrient solutions. Thus, the harmful effect may have been due to the high osmotic concentration of the salt used (0.8 M). Against this view, however, is the stimulation of growth observed with 0.8 M sodium chloride (figure 8), having an osmotic value about two-thirds as great as that of the magnesium chloride. That antagonism between calcium and magnesium does exist for some of the lower organisms has been shown by the work of Lipman and Burgess (1914), Greaves (1920), Brooks (1920a, 1920b), and Lundegardh (1924), cited above.

The possibility of an antagonistic action between sodium and calcium was next considered. To observe the effect of sodium chloride on *Aspergillus niger*, this salt was added to Pfeffer's solution in concentrations varying from 0.1 M to 0.8 M. The results of this experiment may be seen by referring to the graph of figure 8, plotted from the data given in table 9.

TABLE 9

Growth of Aspergillus niger in Pfeffer's solution containing sodium chloride.

CONCENTRATION OF NaCl	DRY WEIGHT OF MYCELIUM[a]
M	*Grams*
Zero	.245[b]
.1	.352
.2	.414
.3	.408
.4	.407
.8	.436

[a] Each value represents the mean for 10 cultures.

[b] Control culture.

It will be noted that sodium chloride, at all tested concentrations, stimulated the growth of the fungus, as indicated by dry weight of mycelium. With increasing concentrations of sodium chloride, the growth curve rises rapidly at first and then more slowly. Although the dry yields are nearly the same throughout a considerable range of concentrations, the greatest dry weight is indicated for the highest tested concentration of sodium chloride—namely, 0.8 M. It is rather surprising that the fungus grew so well in Pfeffer's solution containing such a high concentration of sodium chloride. Spore formation was almost normal at a concentration of 0.1 M

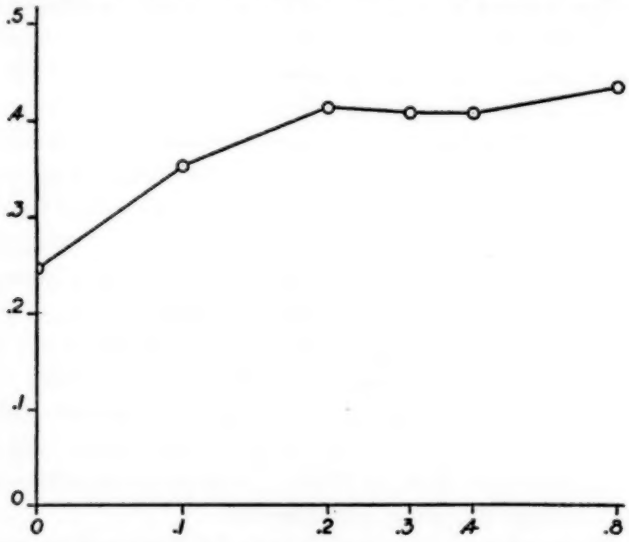

Fig. 8. Dry weights of felts of *Aspergillus niger* grown in Pfeffer's solution to which sodium chloride was added. Abscissas represent molar concentrations of sodium chloride (logarithmic scale). Ordinates represent weights of felts in grams. Control culture without sodium chloride gave yield of 0.245 gram.

sodium chloride and decreased gradually with increasing concentration of the salt, until at 0.4 M the spores were very few in number. There were almost no spores produced at the highest concentration—0.8 M. These results agree in general with those obtained by Molliard (1921), who found that the growth of *Sterigmatocystis nigra* was normal when he added sodium chloride in concentrations of 0.04 M to 0.13 M to a nutrient solution. Few conidia were formed at 0.17 M and none at 0.51 M. The rate of growth diminished as the concentration increased from 0.34 M to 0.86 M; growth was very slow at 1.71 M and was entirely inhibited at 2.05 M. It will be noted, however, that mycelial growth was stimulated in the present tests by concentrations which Molliard found to be toxic.

For studying the combined effects of sodium chloride and calcium chloride in Pfeffer's solution, a series of solutions was employed in which the concentration of sodium chloride was kept uniform, at 0.8 M; this concentration, as just shown, had a marked stimulating effect on mycelial growth. To these solutions, calcium chloride was added in concentrations ranging from zero to 0.1 M.

The results of this experiment may be seen by referring to the graph of figure 9, plotted from the data of table 10. It will be observed that the

TABLE 10

Influence of calcium chloride upon the growth of Aspergillus niger in Pfeffer's solution containing a stimulating concentration of sodium chloride.

CONCENTRATIONS		DRY WEIGHT OF MYCELIUM[a]
NaCl	$CaCl_2$	
M	*M*	*Grams*
Zero	Zero	.292[b]
.8	Zero	.436
.8	.0001	.403
.8	.001	.390
.8	.01	.360
.8	.1	.243

[a] Each value represents the mean for 8 cultures.
[b] Control culture.

curve representing the dry weights of fungus felts drops gradually as the concentration of calcium chloride in the mixed solution increases. Thus, the stimulating effect of sodium chloride on mycelial growth is gradually diminished when calcium chloride is added in increasing concentrations to the solution. A rather abrupt drop in the curve brings the dry weight for the mixture containing 0.1 M calcium chloride to a lower value than that for the control culture (table 10); stimulation has been completely inhibited and this mixture is actually toxic for mycelial growth.

Fruiting of the fungus in the solutions containing calcium chloride and sodium chloride was even less abundant than in the solution to which sodium chloride alone had been added. Conidium formation was completely inhibited in the mixed solution containing 0.1 M calcium chloride.

The question may be raised as to whether the term antagonism should be applied to the effect of one salt in diminishing the stimulation due to another. This term has usually been used with reference to a decrease in toxic action. Calcium chloride, as noted above, reduces the stimulating effect that sodium chloride exerts when added alone to Pfeffer's solution. This is not a case of antagonism in the usual sense; but it is similar to the

action of calcium chloride in decreasing a stimulation of root growth produced by manganese chloride, which was termed antagonism by Barton and Trelease (1927). The results of Lipman (1909, 1910), Winslow and Falk (1923a, 1923b), and Gustafson (1920) on antagonism cannot be compared with the present work, as they were studying lessened toxicity—not diminished stimulation.

The next experiment to be considered is one which was planned in order to determine whether calcium would have any influence upon the

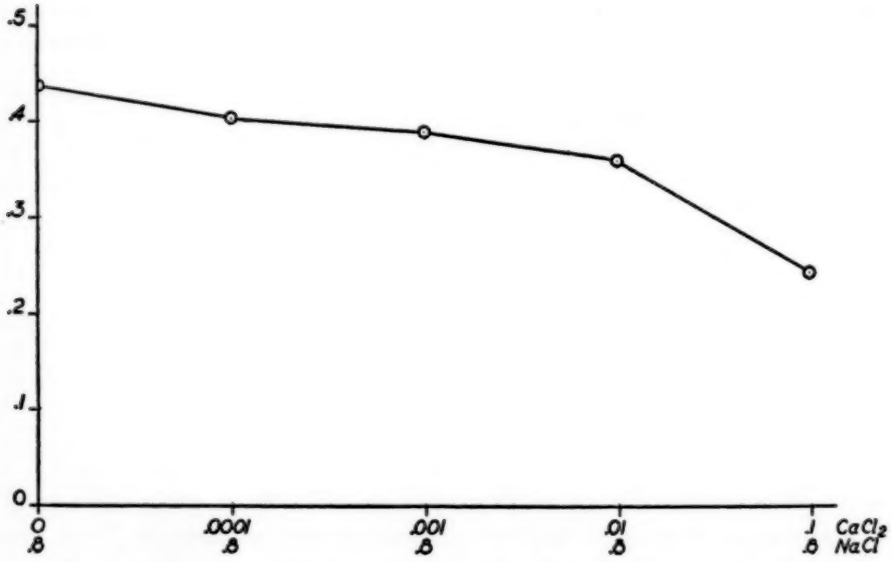

Fig. 9. Dry weights of felts of *Aspergillus niger* grown in Pfeffer's solution containing calcium chloride and sodium chloride. Each culture contained 0.8 M sodium chloride. Abscissas represent molar concentrations of calcium chloride (logarithmic scale). Ordinates represent weights of felts in grams. Control culture without sodium or calcium chlorides gave yield of 0.292 gram.

well known zinc stimulation of *Aspergillus*. Raulin (1869) was the first to observe that zinc sulphate, in very small amounts, has a striking effect in stimulating the mycelial growth of *Aspergillus*, and this phenomenon has since then been observed and studied by many other workers. It is noteworthy that, although the dry weight of the mycelium is greatly increased by minute quantities of zinc, the production of conidia is decreased, frequently to the point of complete inhibition.

In order to see if calcium chloride would have any influence upon this stimulation, *Aspergillus niger* was grown in Pfeffer's solution to which zinc sulphate and this salt were both added. A concentration of 0.00001 M

zinc sulphate was chosen for this experiment because it was found to give a very heavy, wrinkled, white felt, with sparse fruiting; the fungous felt was thick, opaque, and yet brittle. Calcium chloride, in concentrations ranging from zero to 0.2 M, was added to Pfeffer's solution containing this concentration of zinc sulphate. The results of this experiment are presented in the graph of figure 10, plotted from the data given in table 11.

TABLE 11

Influence of calcium chloride upon the growth of Aspergillus niger in Pfeffer's solution contaning a stimulating concentration of zinc sulphate.

CONCENTRATIONS		DRY WEIGHT OF MYCELIUM[a]
$ZnSO_4$	$CaCl_2$	
M	*M*	*Grams*
Zero	Zero	.283[b]
.00001	Zero	.684
.00001	.0125	.446
.00001	.025	.439
.00001	.05	.415
.00001	.1	.373
.00001	.2	.367

[a] Each value represents the mean for 9 cultures.
[b] Control culture.

Inspection of figure 10 shows that when calcium chloride was added to solutions having this concentration of zinc sulphate, the growth stimulation was greatly decreased. Even the addition of the lowest concentration of calcium chloride—namely, 0.0125 M—caused a pronounced drop in the dry weight of the fungus felt. The slope of the curve beyond this point is much more gradual, and even with the highest concentration of calcium chloride the dry weight was above that of the control culture (receiving neither zinc sulphate nor calcium chloride). The felts of the cultures that were supplied with calcium chloride and zinc sulphate were more abnormal in appearance than those to which zinc sulphate alone was added. They were soggy and sank in the liquid, and they exhibited no fruiting.

The combination, then, of zinc sulphate and calcium chloride was not favorable to *Aspergillus*. Calcium chloride alone in Pfeffer's solution had no appreciable effect on the growth or the fruiting of *Aspergillus*. Zinc sulphate alone in Pfeffer's solution greatly stimulated the production of dry material, and in the concentration used (0.00001 M) allowed some fruiting. The mixture of the two salts in Pfeffer's solution, however, had a distinctly harmful effect. The stimulation of mycelial growth was greatly lowered; the felts had an abnormal appearance, and they showed no signs

of sporulation. The study of Hawkins (1913), referred to in the introduction to this paper, involved an inhibition of the germination of the spores of certain fungi by salts of zinc and other metals. Antagonism was definitely shown by the fact that the addition of calcium allowed the spores to germinate. This study cannot be compared to the experiment just described, as zinc in the concentrations used does not inhibit the growth of *Aspergillus*, but on the contrary stimulates it.

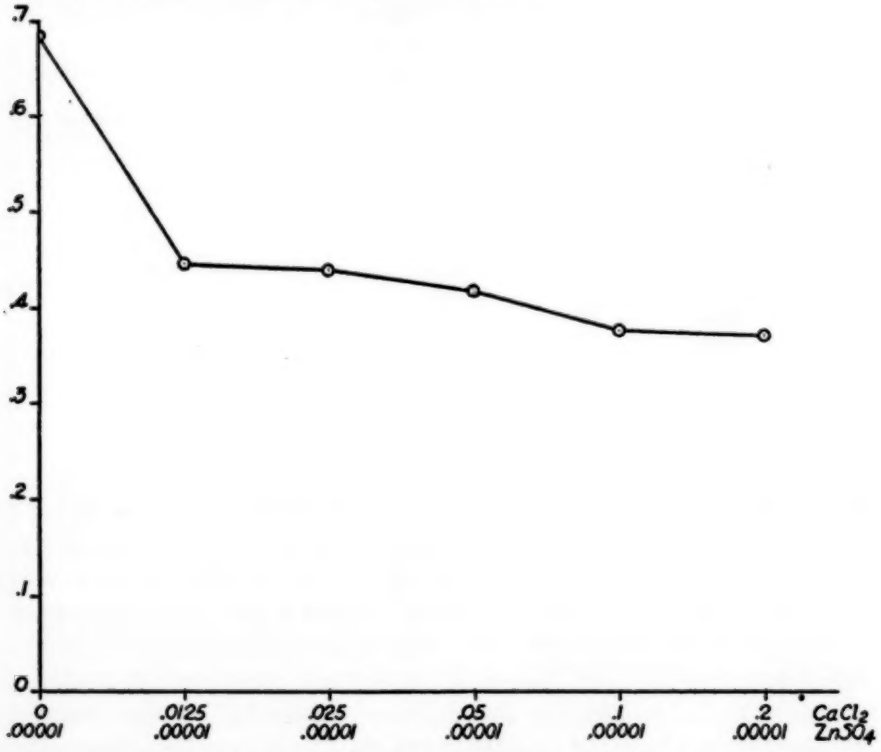

Fig. 10. Dry weights of felts of *Aspergillus niger* grown in Pfeffer's solution containing calcium chloride and zinc sulphate. Each culture contained 0.00001 M zinc sulphate. Abscissas represent molar concentrations of calcium chloride (logarithmic scale). Ordinates represent weights of felts in grams. Control culture without zinc sulphate or calcium chloride gave yield of 0.283 gram.

Influence on the growth of Aspergillus niger of varying the proportions of the three salts of Pfeffer's solution

In the experiments thus far described, the standard Pfeffer's solution was employed in all cases as a basic culture medium. The effects of varying the concentration of magnesium sulphate and of adding salts of calcium and other elements was studied; but no attempt was made to vary in a

systematic manner the proportions of all three component salts of Pfeffer's solution. To obtain further information concerning the salt requirements of *Aspergillus*, it seemed important to study the relations between the growth of the fungus and the proportions of the salts of Pfeffer's solution. The present section of this paper describes three experiments that dealt with this phase of the problem. The first two were concerned with the effects of varying the proportions of the three component salts, and the third involved the influence of calcium chloride upon the physiological values of the culture solutions.

Very few descriptions have appeared in the literature of experiments on fungi in which the proportions of the salts making up a nutrient solution have been varied. The researches along this line carried on by Tottingham (1914) and by Shive (1915) with green plants are so well known that they will not be discussed here. Dunn (1921) and Young and Bennett (1922) performed some similar experiments with fungi. Haenseler (1921) varied the salt proportions of the nutrient solution for *Aspergillus niger*, using calcium nitrate, monopotassium phosphate and magnesium sulphate for the three component nutrient salts; and in a repetition of the experiment he substituted sodium nitrate for calcium nitrate. Haenseler found that the partial concentrations of magnesium sulphate and potassium phosphate could be varied within wide limits without in any way affecting the yields. Steinberg (1919), in a much smaller series of tests, obtained different results. He observed an increased yield with increased amounts of either magnesium sulphate or potassium phosphate.

In the experiments to be described here, the proportions of the three salts of Pfeffer's solution were varied, but the total salt concentration was always kept at 0.1718 M, which is the gram-molecular concentration of the standard solution. The sugar was supplied in the usual concentration of 0.1461 M, or 50 grams per liter. The volume-molecular proportions of the salts for each culture and the corresponding dry weights of the felts are presented in table 12. The table also gives these weights as relative values calculated as a percentage of the highest weight obtained in the series. The equilateral triangle of figure 11 shows each of these percentages placed above and weights placed below the point representing the composition of the solution.

The proportions of the salts are represented on the triangle in the following manner: The upper apex of the triangle represents a solution in which 100 per cent of the dissolved molecules are potassium phosphate. The opposite base represents solutions containing no potassium phosphate. The right apex in the same manner denotes a solution in which 100 per cent of the dissolved molecules are ammonium nitrate, and that on the

left designates one in which 100 per cent are magnesium sulphate. The base opposite each apex represents a solution containing none of the salt represented at the apex. The exact center of the triangle represents a solution containing equal proportions of the three salts, and all other points inside the triangle represent solutions containing mixtures of the three salts. The proportions denoted by any point may be found by measuring the perpendicular distance of the point from each of the three bases.

TABLE 12

Influence on the growth of Aspergillus niger of varying the proportions of the three salts of Pfeffer's solution.

RELATIVE VOLUME-MOLECULAR PROPORTIONS OF SALTS[c]			DRY WEIGHT OF MYCELIUM[a]	
KH_2PO_4	NH_4NO_3	$MgSO_4$	Actual	Relative
			Grams	*Per cent*
5.0	5.0	90.0	.438	94
5.0	47.5	47.5	.321	69
5.0	90.0	5.0	.200	43
15.0	15.0	70.0	.465	100
15.0	70.0	15.0	.293	63
21.4	72.7	5.9	.296[b]	64[b]
33.3	33.3	33.3	.410	88
47.5	5.0	47.5	.374	80
47.5	47.5	5.0	.289	62
70.0	15.0	15.0	.348	75
90.0	5.0	5.0	.277	60

[a] Each value represents the mean for 6 cultures.

[b] Control culture, supplied with same concentrations as in Pfeffer's solution. Value represents the mean of 12 cultures.

[c] Total salt concentration, 0.1718 M.

It may be seen from figure 11 that the highest yields of *Aspergillus niger* (represented by 100 and 94 on the triangle) were obtained with high proportions of magnesium sulphate combined with low proportions of the other two salts. Thus, the growth of the fungus, as indicated by the amount of mycelium formed, was favorably influenced by a high proportion of magnesium sulphate in the nutrient solution. The solution giving the highest weight had the molecular proportions: KH_2PO_4, 15; NH_4NO_3, 15; $MgSO_4$, 70. These figures represent percentages of a total salt concentration of 0.1718 M. The corresponding molecular concentrations of the salts are as follows: KH_2PO_4, 0.02577 M; NH_4NO_3, 0.02577 M; $MgSO_4$, 0.12026 M. The heavy felts produced in this solution were somewhat wrinkled, apparently because there was not room in the flask for such large felts to lie flat; but they were very black and well fruited. The growth was therefore a healthy one.

The growth of the fungus was not favorably influenced by high proportions of ammonium nitrate or of potassium phosphate. The lowest weight of the series, 43 per cent of the best, was obtained in a solution containing the highest tested partial concentration of ammonium nitrate. The next to the lowest weight obtained in this series, 60 per cent of the best, is shown for felts grown in the solution having the highest concentration of potassium phosphate.

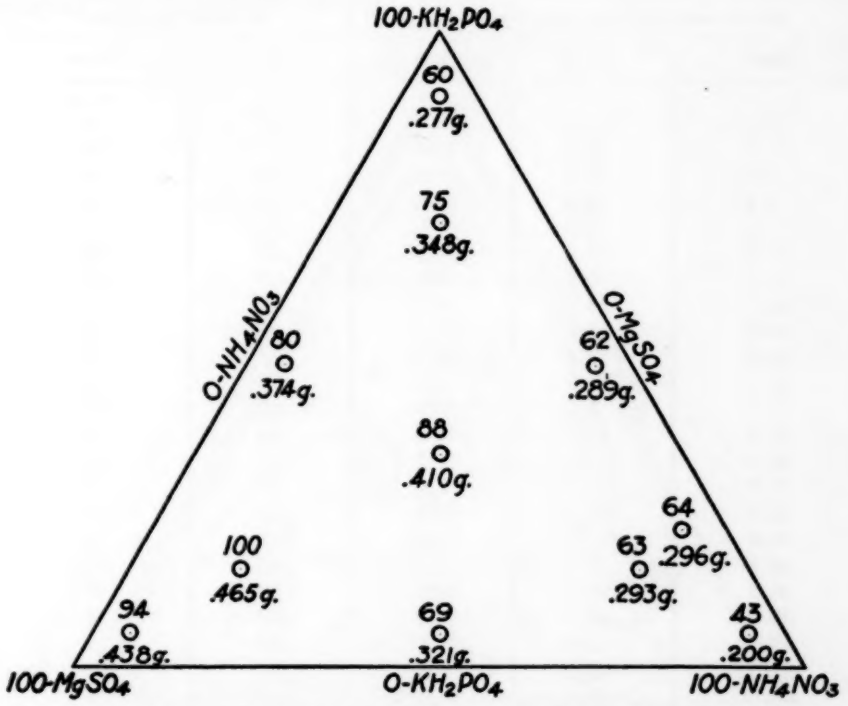

Fig. 11. Relative and actual dry weights of felts of *Aspergillus niger* as influenced by varying the proportions of the three salts of Pfeffer's solution. Upper numbers denote percentages of highest weight taken as 100; lower numbers denote actual weights, for culture solutions whose compositions are indicated by the positions of the points in the triangle.

It will be noticed that the control culture, containing the three salts in the same proportions as in Pfeffer's solution, gave only 64 per cent of the best growth. This solution has only a slightly higher proportion of potassium phosphate than the solution giving the best growth. It is characterized, however, by a much lower proportion of magnesium sulphate and a much higher proportion of ammonium nitrate. The low yield of this culture in comparison with that of the best culture indicates the marked

improvement that can be brought about by altering the salt proportions of Pfeffer's solution. It is clear that a solution containing more magnesium sulphate and less ammonium nitrate than are to be found in Pfeffer's solution gives much better growth of *Aspergillus niger* than is given with the

TABLE 13

Influence on the growth of Aspergillus niger of varying the proportions of the three salts of Pfeffer's solution.

RELATIVE VOLUME-MOLECULAR PROPORTIONS OF SALTS[c]			DRY WEIGHT OF MYCELIUM[a]	
KH_2PO_4	NH_4NO_3	$MgSO_4$	Actual	Relative
			Grams	*Per cent*
5.0	5.0	90.0	.615	92
5.0	25.0	70.0	.669	100
5.0	47.5	47.5	.561	84
5.0	70.0	25.0	.410	61
5.0	90.0	5.0	.245	37
15.0	15.0	70.0	.648	97
15.0	30.0	55.0	.615	92
15.0	55.0	30.0	.388	58
15.0	70.0	15.0	.298	44
20.0	40.0	40.0	.527	79
21.4	72.7	5.9	.347[b]	52[b]
25.0	5.0	70.0	.508	76
25.0	25.0	50.0	.466	70
25.0	50.0	25.0	.406	61
25.0	70.0	5.0	.297	43
30.0	15.0	55.0	.500	75
30.0	55.0	15.0	.360	54
33.3	33.3	33.3	.453	68
40.0	20.0	40.0	.540	81
40.0	40.0	20.0	.387	58
47.5	5.0	47.5	.449	67
47.5	47.5	5.0	.391	57
50.0	25.0	25.0	.396	59
55.0	15.0	30.0	.412	61
55.0	30.0	15.0	.459	69
70.0	5.0	25.0	.395	59
70.0	15.0	15.0	.444	66
70.0	25.0	5.0	.443	66
90.0	5.0	5.0	.300	45

[a] Each value represents the mean for 2 cultures.
[b] Control culture, supplied with same concentration as in Pfeffer's solution.
[c] Total salt concentration, 0.1718 M.

use of Pfeffer's solution. The amount of potassium phosphate, however, in this solution seems to be favorable.

The results of Dunn (1921) working with plus and minus strains of *Rhizopus* were markedly different from these. The solution in which she

obtained the best growth of both strains contained about equal molecular proportions of ammonium nitrate and magnesium sulphate, and a partial concentration of monopotassium phosphate about six times as great as that of either of the other two. Steinberg (1919) thought that the increased yield which he observed with large amounts of magnesium sulphate was due to iron impurities in this salt; but Currie (1917) found that iron did not increase the growth of *Aspergillus niger* when the nutrient solution was supplied with ammonium nitrate.

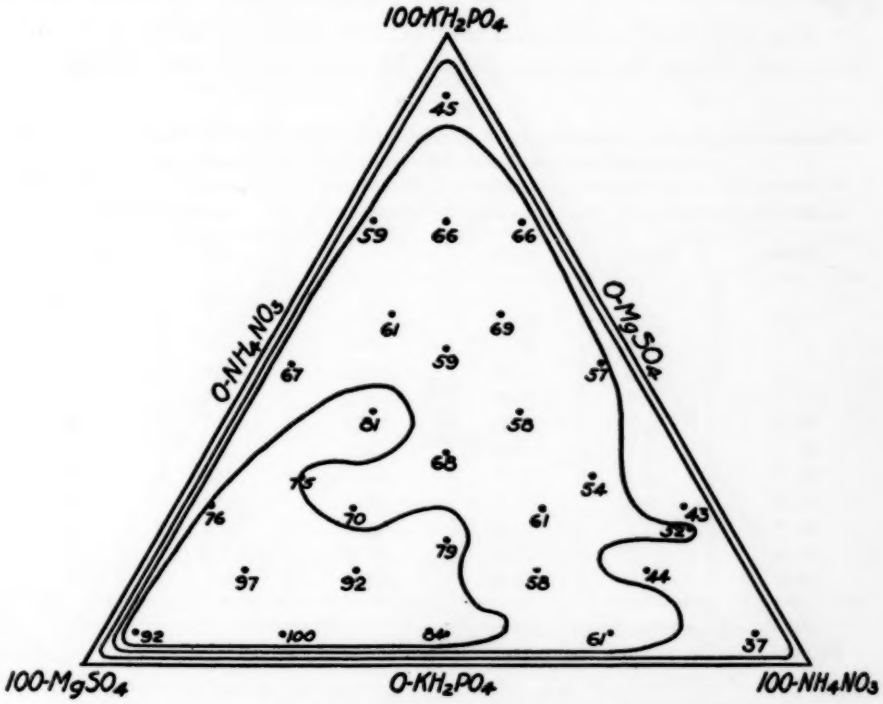

Fig. 12. Relative weights of felts of *Aspergillus niger* as influenced by varying the proportions of the three salts of Pfeffer's solution. Numbers denote percentages of highest weight taken as 100. Points in triangle indicate compositions of culture solutions. Contour lines divide area of triangle into the following zones: 0 to 25; 25 to 50; 50 to 75; 75 to 100.

The experiment just described was repeated on a more extensive scale by means of a duplicated series in which twenty-nine different sets of salt proportions were tested. These included eighteen new solutions in addition to the eleven that had previously been employed. The results of this experiment appear in table 13 and are represented graphically in the triangle of figure 12.

After expressing the dry weights of the felts as percentages of the heaviest, the resulting relative weights were divided into four groups: (1) from 75 to 100 per cent of the heaviest, (2) from 50 to 75, (3) from 25 to 50, and (4) from zero to 25. Contour lines were drawn on the triangle to include the ranges of values just indicated. The two outer zones of course are only rough approximations. In drawing the contour line that separates them it was assumed that each of the three sides of the triangle would represent zero growth; this seemed justified since it had been found by experiment that *Aspergillus* gave no growth if supplied with only two of the salts of Pfeffer's solution. The other two zones, however, seem to be fairly well defined by the numerous yield values which they include.

TABLE 14

Influence on the growth of Aspergillus niger of varying the proportions of the three salts of Pfeffer's solution with calcium chloride present in addition to the three salts.

RELATIVE VOLUME-MOLECULAR PROPORTIONS OF SALTS[c]			CONCENTRATION OF $CaCl_2$	DRY WEIGHT OF MYCELIUM[a]	
KH_2PO_4	NH_4NO_3	$MgSO_4$		Actual	Relative
			M	*Grams*	*Per cent*
5.0	5.0	90.0	.0625	.503	93
5.0	47.5	47.5	.0625	.359	67
5.0	90.0	5.0	.0625	.255	47
15.0	15.0	70.0	.0625	.539	100
15.0	70.0	15.0	.0625	.307	57
21.4	72.7	5.9	Zero	.291[b]	54[b]
33.3	33.3	33.3	.0625	.366	68
47.5	5.0	47.5	.0625	.256	48
47.5	47.5	5.0	.0625	.255	47
70.0	15.0	15.0	.0625	.323	60
90.0	5.0	5.0	.0625	.203	38

[a] Each value represents the mean for 6 cultures.

[b] Control culture, supplied with same concentrations as in Pfeffer's solution. Value represents the mean for 12 cultures.

[c] Total salt concentration without $CaCl_2$, 0.1718 M.

It will be noticed that the zone of high yields is in the lower left-hand corner of the triangle, corresponding to relatively high proportions of magnesium sulphate combined with low and medium proportions of the other two salts. The distribution of dry yields exhibits in general a satisfactory agreement with that obtained in the preceding experiment. The highest yield (indicated by 100 in the triangle) was obtained with a solution which had not been used in the preceding experiment; it contained less potassium phosphate than the best solution of the former experiment. But the solution that had been best in the preceding experiment gave a relative yield of 97 in the present experiment. The difference between the values 100

and 97 is so small that it is probably not significant. The control culture, supplied with the standard Pfeffer's solution, had a relative dry yield of only 52 per cent of that secured with the best solution. The results of this more extensive experiment show clearly, as do those represented graphically in figure 11, that the proportions of Pfeffer's solution can be improved for the growth of *Aspergillus* by increasing the partial concentration of magnesium sulphate and decreasing that of ammonium nitrate.

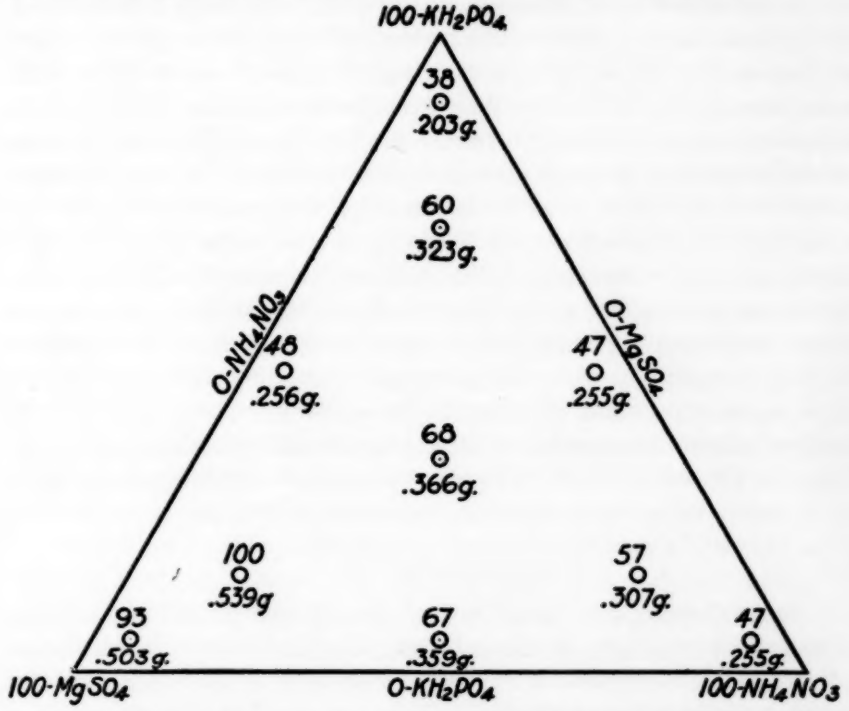

Fig. 13. Relative and actual dry weights of felts of *Aspergillus niger* as influenced by varying the proportions of the three salts of Pfeffer's solution, when each culture solution contained 0.0625 M calcium chloride. Upper numbers denote percentages of highest weight taken as 100; lower numbers denote actual weights, for culture solutions whose compositions are indicated by the positions of the points in the triangle.

A third series of cultures was conducted in an attempt to determine whether the addition of calcium chloride to the culture solutions would influence the distribution of dry yields, as these are related to the molecular proportions of the three salts of Pfeffer's solution. For this purpose calcium chloride in a concentration of 0.0625 M was added to each of ten culture solutions in which the proportions of the salts were varied in the same way as in the first experiment. The results of growing *Aspergillus* in these solutions are given in table 14 and are shown graphically in the

triangle of figure 13. A comparison of the three sets of triangular graphs—the first two for solutions lacking calcium and the last for those containing this element—fails to reveal any differences, either in the distribution of yields or in their absolute values, that can with certainty be ascribed to the influence of calcium. Very high dry yields were obtained in the lower left-hand corner of the triangle, both in the presence and in the absence of calcium. Very low yields are indicated for the lower right-hand corner and for the upper apex, with and without calcium. Such differences as exist between the dry yields of the corresponding cultures appear to have been due to the variability of the biological material rather than to the presence or absence of calcium. Variations between the two series lacking calcium are as great as those between either of these series and the series containing calcium. In general the comparison of these three experiments leads to the conclusion that calcium had no very pronounced influence on the responses of the fungus to the main nutrient salts. The presence of calcium chloride in the culture solution seemed to cause the fruiting to appear brownish in color, rather than black; hence calcium may tend to increase the formation of the yellow pigment often seen in the hyphae of this fungus, especially when the spores germinate on bread. Some of this yellow pigment probably dissolves in the nutrient solution, giving it the characteristic yellow appearance caused by the addition of a calcium salt. Thom and Church (1926, p. 11) point out a relation between the production of yellow colors and acid conditions of the substrata.

SUMMARY

Aspergillus niger and *Penicillium sp.* were grown in Pfeffer's solution and in certain variations of this solution. The fungi were cultured in 150 cc. Pyrex flasks, *Aspergillus* in an incubator at 34°C. for 5 days, *Penicillium* at a room temperature of about 22°C. for 6 days. The felts were placed on weighed filter papers, dried at 65° to 70°C. for at least 3 days and weighed. The weights of the felts and the appearance of the cultures, particularly with respect to conidium formation, were used as the basis for a comparison of the physiological effects of the culture solutions. The principal conclusions follow:

The weights of the felts alone could not be taken as a satisfactory criterion of the development of these fungi without also taking into consideration their fruiting.

Magnesium was found to be absolutely essential for the growth of both *Aspergillus* and *Penicillium*. This element must be required for some important but as yet unknown physiological function; and for good growth of the fungi it must be supplied in concentrations higher than 0.0001 gram molecule per liter of the culture solution. Though obviously not required

for the production of chlorophyll in these fungi, magnesium is as indispensable for them as for the higher green plants. High concentrations of magnesium poison *Aspergillus* as they do the higher plants, but the minimum toxic concentration is greater for the fungus.

Spectrographic analysis showed that some calcium was present in the mycelium of *Aspergillus* grown in Pfeffer's solution that had been prepared with doubly distilled water and the best grade of salts obtainable. The fungus was evidently able to absorb small amounts of calcium which were supplied as an impurity in the mineral salts or sugar, or it derived these salts from the spores. Precipitation tests with sodium oxalate showed that the culture solutions contained less than 0.000001 M of a salt of calcium—that is, less than 1 part of calcium per 25 million parts of the culture medium. *Aspergillus* also was found to make excellent growth in a culture solution containing a very high concentration (0.1 M) of sodium oxalate, which presumably would precipitate all but exceedingly minute traces of calcium. These considerations lead to the conclusion that if calcium is essential for the development of *Aspergillus* it is needed only in extremely small quantities; the amount of calcium required—if any—is no greater than the quantity of boron, zinc, manganese, iron, or copper that is essential for the higher green plants and possibly also for the fungi.

Calcium chloride did not antagonize the poisoning of *Aspergillus* due to high concentrations of magnesium chloride in the culture solution. In a concentration of 0.1 M, it increased the toxic effect.

A stimulation of mycelial growth that resulted from addition of sodium chloride to the culture medium was decreased when calcium chloride was also added; but the addition of both of these salts produced felts which were more abnormal in appearance than those obtained by the addition of either salt alone to Pfeffer's solution.

Calcium chloride decreased the well-known stimulation of mycelial growth due to zinc sulphate; but the addition of these two salts had a decidedly toxic effect on the fungus felts, spore formation being suppressed much more than with either salt alone in Pfeffer's solution.

A marked improvement over the standard salt proportions of Pfeffer's solution was obtained by varying the three salts in a systematic manner. The growth of *Aspergillus niger* was greatest in solutions that contained higher proportions of magnesium sulphate and lower proportions of ammonium nitrate than those of Pfeffer's solution. The addition of a calcium salt seemed to have no pronounced influence on the growth responses of *Aspergillus* to the three main salts.

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The anatomy of the leaf of Panicum palmifolium

TERZO P. AMIDEI

(WITH THREE TEXT FIGURES)

In general every foliage leaf of the grass plant consists of two parts, the blade and the sheath. The ligule, which varies in size and shape, also occurs in most grass leaves. A petiole-like stalk is inserted between the blade and the sheath in the leaves of a few tropical and semi-tropical grasses, such as *Pharus, Phyllorachis, Olyra, Ischaemum,* many of the *Bambusae* and some of the species of *Panicum* (Hackel, 1890). The leaves of many of these plants have broad plicate blades.

The above mentioned features of the grass leaf are all exemplified in the leaf of *Panicum palmifolium* Willd.,[1] plants of which have been successfully grown in the greenhouse at Indiana University for many years. Because of the ease with which the plant can be grown, I have been able to get for this study all stages in the development from the seed to the adult plant.

The peculiar features of the leaf give rise to many questions, of which the following are examples: (1) Is the stalk of the leaf a real petiole? (2) What is the mode of development and the internal effect of the corrugations of the leaf? (3) What are the functions of the corrugations? (4) Is the venation of the leaf really pinnate? (5) How is the corrugated leaf related phylogenetically to the ordinary flat leaf of grasses?

LITERATURE

In the extensive literature on the anatomy of the grass leaf, some of these questions have already been considered as applied to other grasses. Duval-Jouve (1875) gives a summary of the work done up to 1865 making special mention of Scheuzcher, Babel and P. de Beauvois. Scheuzcher was the first botanist to divide the grass leaf into blade, sheath and ligule. P. de Beauvois (1822) supported a theory that Linnaeus set forth in the thesis of his student, H. Gahn. Linnaeus thought that all the leaves of grasses were similar in structure. This idea, resulting from insufficient study on the part of de Beauvois, hindered the investigations along this line for a long time. In 1865, Wiess saw the openings of the stomata and a few years later Pfitzer tried to explain the function and the make-up of the stomata.

Duval-Jouve was primarily interested in finding anatomical characteristics which would have taxonomic value. Pée-Laby (1898) carried this idea a step farther, attempting as he says in the introduction, to group the

[1] *Chaetochloa palmifolia* (Willd.) Hitchc. & Chase.

grasses of France according to taxonomic characters of the leaves and to make known the physiological rôle of certain foliar tissues based upon the arrangement, the structure, and the development of their elements. The work done since 1865 on the grass leaf is summarized by Löv (1926) in a paper on the mechanism of the unfolding of the leaves of monocotyledonous plants.

GENERAL FORM OF THE LEAF

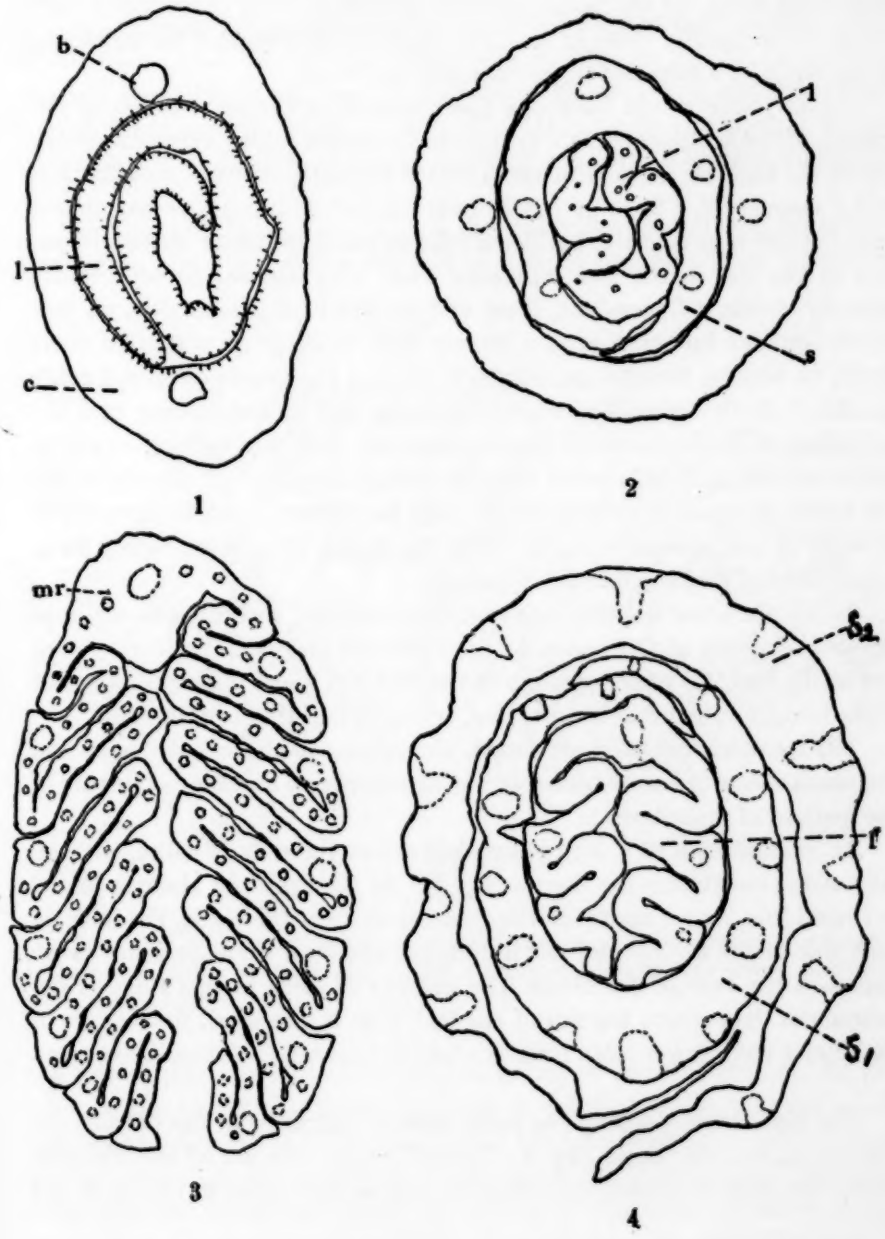
At the present time, there are various theories as to the anatomical significance of the parts of the grass leaf. De Candolle's phyllode theory, also supported by Arber (1918), maintains that the monocotyledonous leaf does not correspond to the complete dicotyledonous leaf with its base, stipules, blade, and petiole, but is merely equivalent to the petiole and the sheathing base. Another theory championed by Bugnon (1921) holds that the blade is equivalent to the basal sheath of the leaf of the dicotyledon and that the sheath of the grass leaf is a new structure. A third and more common theory is that the parts of the grass leaf are the same as those of the dicotyledonous leaf; the sheath of the grass leaf is equivalent to the leaf base, and the ligule to the stipules; the blade is the same in both and the stalk present in some grass leaves is a true petiole (Hitchcock, 1922). It is not my purpose to give further evidence on the theories, but I shall use the nomenclature of the last mentioned.

The leaves of *Panicum palmifolium* have four distinct parts, sheath, petiole, ligule and blade. The broad blade tapers towards both ends to a general oblanceolate form. The upper surface is covered with fine velvety hair and the margin is minutely serrate. Its most striking feature is the series of oblique longitudinal folds which are especially prominent when the plant has insufficient moisture. There is a prominent midrib, and it continues in some of the leaves as a distinct petiole. In the case of the young plants, the petiole may be from two to four inches long but in the leaves formed later it is very short or entirely lacking. The ligule is made up of long hairs. The sheath is much like that of the other grasses.

The oldest tissues of a grass leaf are at the top and growth continues at the base long after the tip is mature. The fact that the leaf has a petiole, blade, sheath, and ligule that are so different from one another, raises the question as to how all of them could have grown from a single primordium in the bud. No attempt to answer this question will be made here.

THE CORRUGATIONS OF THE BLADE

The development and the functions of the corrugations of the leaves of this kind have received attention from many workers. When the leaf emerges from the bud, it is folded flat as a fan and expands by a hinge ac-



Figs. 1–4. 1. Cross section of a seedling showing juvenile leaf. *b*, fibrovascular bundle; *c*, coleoptile; *l*, juvenile leaf. Fig. 2. Cross section of a seedling showing the first folded leaf. *l*, first folded leaf; *s*, sheath of the juvenile leaf. Fig. 3. Cross section of a much later leaf still in the bud. The sheaths on the outside removed; *mr*, midrib. Fig. 4. Cross section of seedling showing third folded leaf. s_2 sheath of first folded leaf; s_1 sheath of second folded leaf; *l*, third folded leaf.

tion of the folds. At the bottom of each groove is a line of bulliform cells which are ordinarily thought to bring about the folding and the unfolding of the blade by a response to the change of moisture.

Duval-Jouve would have one think that it is the retardation of the growth of the bulliform cells which gives rise to the folded vernation of the leaf in the bud. He finds evidence of this in the comparison of an adult leaf and a young leaf. Pée-Laby (1898) believes that Duval-Jouve has gone a little too far in giving the bulliform cells so much influence on the vernation of the leaf in the bud especially when they are not present or are scarcely visible. "Cependant, il est allé un peu loin, à mon avis, en leur attribuant une influence prépondérante dans la mode de vernation de la feuille en bouton, lorsque ces cellules n'existent pas encore ou sont à peine sensible." Both Duval-Jouve and Pée-Laby tell of the folding and the unfolding of the leaves when they mature, but they say nothing as to the initial unfolding. Löv believes that the initial expansion of the leaf is not the result of equal growth of all the cells but rather a rapid enlargement of more or less specialized cells which up to the time of unfolding have lagged behind the others in development.

To obtain some definite views on this problem, a study was made of the development of the leaves at five different stages: (1) a very young leaf in the bud, (2) an older stage in the bud, (3) a leaf that is coming out of the bud, (4) a leaf that is unfolding, and (5) a leaf that is unfolded.

The material was fixed, sectioned, and stained in the ordinary way and the results obtained were probably not dependent on any special feature of the method of procedure.

An examination of a very young leaf shows the cells all alike, with no differentiation among the tissues (fig. 2).[1] At the apex the blade is folded in the middle. As we approach the greatest width of the blade, the folds on each side of the midrib increase in number and then decrease again as we approach the base of the blade. The number of folds at this stage of development depends on the size of the leaf, that is, the small first leaves of the plants have fewer folds than the larger ones which follow them (figs. 3, 4).

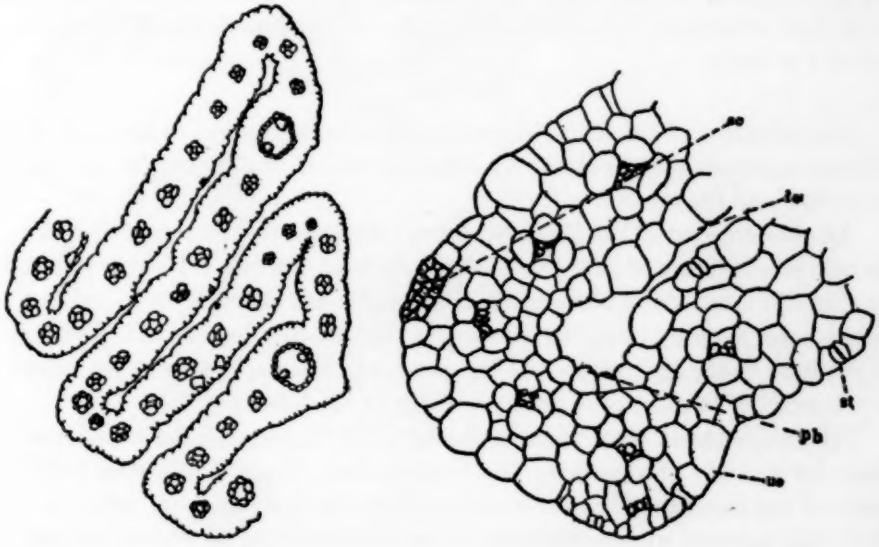
The older leaves, still in the buds, show a beginning of the differentiation of the vascular tissue (fig. 4). The epidermal cells are all alike in contents; the only difference that is noticeable is that those in the folds are smaller.

When a portion of the leaf emerges from the bud, it shows a complete differentiation of all the main tissues (fig. 3). The epidermal cells are well

[1] All the figures pertain to *Panicum palmifolium* Willd. They are camera lucida drawings from microtome sections.

developed except for those that are found in the folds. These are still small and full of dense protoplasm (fig. 6). It will be noticed that there are two folds between each two adjacent large primary bundles (fig. 5). I am of the opinion that this is the reason that both Duval-Jouve and Pée-Laby thought they saw the bulliform cells. What they really saw was the group of small cells at the base of the folds, which are later to become bulliform cells.

The first step in the unfolding of the leaf is the spreading apart of the two halves (fig. 7); this is a mechanical process that will occur whenever



5 6

Figs. 5, 6. 5. A portion of fig. 3 enlarged. Fig. 6. A portion of fig. 5 enlarged; *le*, lower epidermis; *pb*, primodial bulliform cells; *sc*, sclerenchyma tissue; *st*, stoma; *ue*, upper epidermis.

the leaf obtains enough room. The other folds are still present. In the parts of the leaves that have unfolded, it will be noticed that the small cells which were in the folds have grown fourfold to tenfold making them much larger than the rest of the epidermal cells; these are the bulliform cells. The leaf is not regular in its method of unfolding. In some of the leaves it begins in the center and in others at the ends; and sometimes it is somewhere between. Though there is no regularity in the unfolding, the bulliform cells of the upper epidermis develop before the corresponding cells of the lower epidermis. The irregularity in the process of the unfolding

should be studied more carefully and more extensively before any conclusions are to be drawn from it.

A section of the adult leaf shows the typical ridges and grooves (figs. 8–11). How well defined the ridges and grooves will be depends on the condition of the plant when the leaves were fixed. A leaf that has fully turgid bulliform cells will be almost flat (fig. 11).

It is certain that the initial folds and the initial unfolding are not caused by the hygroscopic sensitiveness of the bulliform cells. The initial folds must be attributed to the crowded conditions in the bud and the initial unfolding to the rapid development of the bulliform cells which have been retarded in the limited space at the bottom of the grooves between the folds.

VENATION

The venation of the leaf is of interest because it appears to be pinnate. The corrugations extend obliquely from the midrib to the margin and the veins run into the midrib.

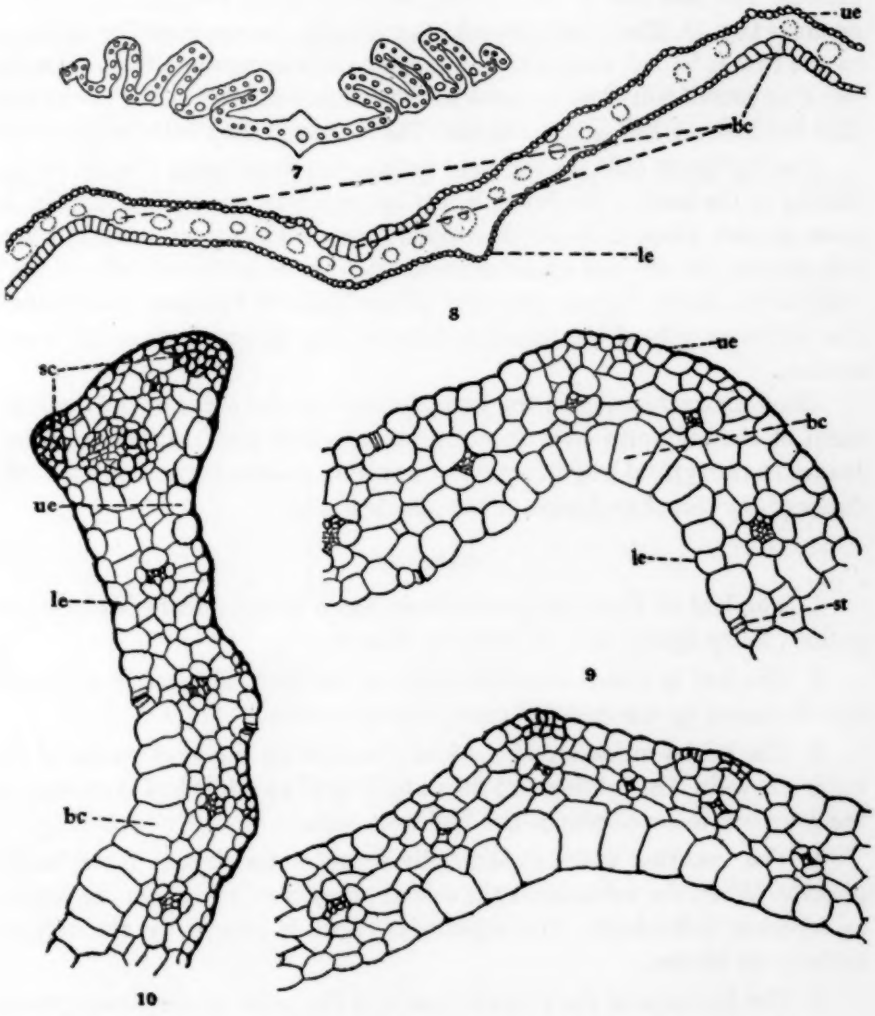
An examination of the sections shows that after the vein runs into the midrib it continues down the midrib without uniting with the other bundles. In a section of the midrib, the bundles are arranged in a crescent on the lower side. By counting, it is found that the number of main bundles at the base of the leaf, where the leaf is mostly petiole, is almost the same as the number found at the greatest width of the blade.

Several methods were tried in the attempt to prepare the whole leaf blade for a study of venation. The method that Evans (1928) used in his study of the bundles of the stem of *Zea Mays* did not prove successful because the amount of sclerenchyma makes the process of retting almost impossible. The best method found was to decolorize, stain and clear the leaf and then to examine at low magnification. The leaves were decolorized with alcohol and placed in anilin safranin for twelve or more hours. After sufficient destaining and dehydration with alcohol, they were gradually changed to xylol.

On the examination of these leaves, it was found that the veins were almost parallel to each other, and that most of them went into the midrib, and from there they went down the midrib as separate individuals. At the base of the blade a few veins were found to unite with others instead of going separately into the midrib. This is an indication of a tendency towards true pinnate venation. Besides these there are found in the leaf blade a great number of veinlets that merely provide cross connections between the main veins. In spite of these lateral unions of veins it seems that we have only a slight modification of the parallel venation common in other grasses.

PHYLOGENY

The folded leaf blade is an adaptation of the plant to a definite set of conditions. When the leaf is still in the bud, it is necessary that it be folded



Figs. 7–11. 7. A cross section of a leaf that has emerged. Fig. 8. A portion of a cross section of a blade showing three groups of bulliform cells; *bc*, bulliform cells; *le*, lower epidermis; *ue*, upper epidermis. Fig. 9. Cross section of blade showing a group of bulliform cells. *bc*, bulliform cells; *le*, lower epidermis; *st*, stoma; *ue*, upper epidermis. Fig. 10. Cross section of the edge of the blade. *bc*, bulliform cells; *le*, lower epidermis; *sc*, sclerenchyma tissue; *ue*, upper epidermis. Fig. 11. Cross section of the blade showing a group of turgid bulliform cells.

to be accomodated to the small space. When it is fully developed the folds hold the blade rigid and prevent too rapid transpiration.

The folded leaf seems to be a characteristic of recent evolution as is seen in the seedling. The plant starts its growth like all other species of grasses. The first leaf of the seedling is rolled in the bud but flat after it emerges (fig. 1). The successive leaves gradually change from the flat type to one that is folded, each leaf being wider and having more folds than the one that precedes it. The increase in the number of folds is due to the fact that the broader leaf is in a bud that has increased very little in diameter.

The bulliform cells, as was said before, develop during the initial unfolding of the leaves; the juvenile leaf never forms any bulliform cells. In most grasses, these cells are developed only on the upper surface; but a few species, like the one under consideration, have bulliform cells on both sides of the blade. In any one part of the blade of *Panicum palmifolium* the bulliform cells of the upper surface develop before those of the lower surface.

These characteristics of the juvenile leaf and the order of the development of the bulliform cells do not *prove* anything but their close resemblance to the typical leaf structure of common grasses is cited as good evidence of the recent evolution of the plicate blade.

SUMMARY

1. The leaf of *Panicum palmifolium* has a broad plicate blade, short petiole, hairy ligule, and an ordinary sheath.

2. The leaf is folded conduplicately in the bud. This mode of vernation is caused by the crowded conditions that exist.

3. The initial unfolding of the leaf is caused by the *development* of the bulliform cells. The folding and the unfolding of an adult leaf is caused by the *hygroscopic sensitivity* of the bulliform cells.

4. The venation which superficially seems to be pinnate is not really pinnate. When the veins enter the midrib, they continue down the midrib as separate individuals. The superficial effect is caused by the oblique folds of the blades.

5. The features of the juvenile leaf and the order of the development of the bulliform cells seem to indicate that the plicate leaf is of recent origin.

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INDEX TO AMERICAN BOTANICAL LITERATURE
1931–1932

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